

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
3 May 2001 (03.05.2001)

PCT

(10) International Publication Number
WO 01/31035 A2

(51) International Patent Classification⁷: C12N 15/61, 9/90, 1/19, 1/21, C12P 11/00, 19/62 // (C12P 11/00, C12R 1:19) (C12P 19/62, C12R 1:19) (C12N 1/21, C12R 1:19) (C12P 11/00, C12R 1:865) (C12P 19/62, C12R 1:865) (C12N 1/19, C12R 1:865)

Street, San Carlos, CA 94070 (US). DAYEM, Linda; 2240 Village Court, #2, Belmont, CA 94002 (US). KEALEY, James; 19 Watt Avenue, San Rafael, CA 94901 (US).

(21) International Application Number: PCT/US00/29775

(74) Agents: FAVORITO, Carolyn, A. et al.; Morrison & Forster LLP, 3811 Valley Centre Drive, Suite 500, San Diego, CA 92130-2332 (US).

(22) International Filing Date: 27 October 2000 (27.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/161,703 27 October 1999 (27.10.1999) US

(71) Applicant: KOSAN BIOSCIENCES, INC. [US/US];
3832 Bay Center Place, Hayward, CA 94545 (US).

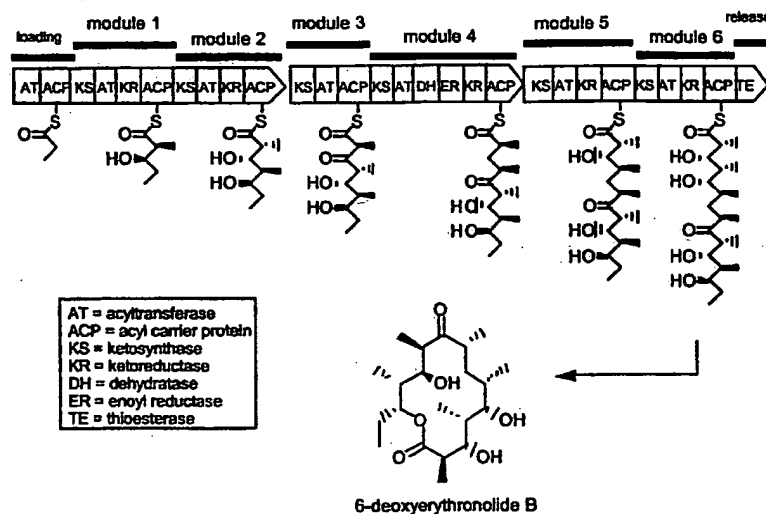
(72) Inventors: SANTI, Daniel; 211 Belgrave Avenue, San Francisco, CA 94117 (US). PECK, Larry; 910 Laurel

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: HETEROLOGOUS PRODUCTION OF POLYKETIDES



Biosynthesis of 6-Deoxyerythronolide B (6-dEB), the Aglycone of Erythromycin, by a Modular PKS

(57) Abstract: Recombinant host cells that comprise recombinant DNA expression vectors that drive expression of a product and a precursor for biosynthesis of that product can be used to produce useful products such as polyketides in host cells that do not naturally produce the product or produce the product or precursor at low levels due to the absence of the precursor or the presence of the precursor in rate limiting amounts.



Published:

— Without international search report and to be republished upon receipt of that report.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Title

Heterologous Production of Polyketides

Field of the Invention

5 The present invention provides recombinant methods and materials for producing polyketides by recombinant DNA technology. The invention relates to the fields of agriculture, animal husbandry, chemistry, medicinal chemistry, medicine, molecular biology, pharmacology, and veterinary technology.

10

Background of the Invention

Polyketides represent a large family of diverse compounds synthesized from 2-carbon units through a series of condensations and subsequent modifications. Polyketides occur in many types of organisms, including fungi and mycelial bacteria, in particular, the actinomycetes. There are a wide variety of polyketide structures, and the class of polyketides encompasses numerous compounds with diverse activities. Erythromycin, FK-506, FK-520, megalomicin, narbomycin, oleandomycin, picromycin, rapamycin, spinocyn, and tylosin are examples of such compounds. Given the difficulty in producing polyketide compounds by traditional chemical methodology, and the typically low production of polyketides in wild-type cells, there has been considerable interest in finding improved or alternate means to produce polyketide compounds. See PCT publication Nos. WO 93/13663; WO 95/08548; WO 96/40968; 97/02358; and 98/27203; United States Patent Nos. 4,874,748; 5,063,155; 5,098,837; 5,149,639; 5,672,491; 5,712,146; and 5,962,290; and Fu *et al.*, 1994, *Biochemistry* 33: 9321-9326; McDaniel *et al.*, 1993, *Science* 262: 1546-1550; and Rohr, 1995, *Angew. Chem. Int. Ed. Engl.* 34(8): 881-888, each of which is incorporated herein by reference.

Polyketides are synthesized in nature by polyketide synthase (PKS) enzymes. These enzymes, which are complexes of multiple large proteins, are similar to the synthases that catalyze condensation of 2-carbon units in the

biosynthesis of fatty acids. PKS enzymes are encoded by PKS genes that usually consist of three or more open reading frames (ORFs). Two major types of PKS enzymes are known; these differ in their composition and mode of synthesis. These two major types of PKS enzymes are commonly referred to as Type I or "modular" and Type II "iterative" PKS enzymes. A third type of PKS found primarily in fungal cells has features of both the Type I and Type II enzymes and is referred to as a "fungal" PKS enzymes.

Modular PKSs are responsible for producing a large number of 12-, 14-, and 16-membered macrolide antibiotics including erythromycin, megalomicin, methymycin, narbomycin, oleandomycin, picromycin, and tylosin. Each ORF of a modular PKS can comprise one, two, or more "modules" of ketosynthase activity, each module of which consists of at least two (if a loading module) and more typically three (for the simplest extender module) or more enzymatic activities or "domains." These large multifunctional enzymes (>300,000 kDa) catalyze the biosynthesis of polyketide macrolactones through multistep pathways involving decarboxylative condensations between acyl thioesters followed by cycles of varying β -carbon processing activities (see O'Hagan, D. *The polyketide metabolites*; E. Horwood: New York, 1991, incorporated herein by reference).

During the past half decade, the study of modular PKS function and specificity has been greatly facilitated by the plasmid-based *Streptomyces coelicolor* expression system developed with the 6-deoxyerythronolide B (6-dEB) synthase (DEBS) genes (see Kao *et al.*, 1994, *Science*, 265: 509-512, McDaniel *et al.*, 1993, *Science* 262: 1546-1557, and U.S. Patent Nos. 5,672,491 and 5,712,146, each of which is incorporated herein by reference). The advantages to this plasmid-based genetic system for DEBS are that it overcomes the tedious and limited techniques for manipulating the natural DEBS host organism, *Saccharopolyspora erythraea*, allows more facile construction of recombinant PKSs, and reduces the complexity of PKS analysis by providing a "clean" host background. This system also expedited construction of the first combinatorial modular polyketide library in

Streptomyces (see PCT publication Nos. WO 98/49315 and 00/024907, each of which is incorporated herein by reference).

The ability to control aspects of polyketide biosynthesis, such as monomer selection and degree of β -carbon processing, by genetic manipulation of PKSs has stimulated great interest in the combinatorial engineering of novel antibiotics (see Hutchinson, 1998, *Curr. Opin. Microbiol.* 1: 319-329; Carreras and Santi, 1998, *Curr. Opin. Biotech.* 9: 403-411; and U.S. Patent Nos. 5,712,146 and 5,672,491, each of which is incorporated herein by reference). This interest has resulted in the cloning, analysis, and manipulation by recombinant DNA technology of genes that encode PKS enzymes. The resulting technology allows one to manipulate a known PKS gene cluster either to produce the polyketide synthesized by that PKS at higher levels than occur in nature or in hosts that otherwise do not produce the polyketide. The technology also allows one to produce molecules that are structurally related to, but distinct from, the polyketides produced from known PKS gene clusters.

There has been a great deal of interest in expressing polyketides produced by Type I and Type II PKS enzymes in host cells that do not normally express such enzymes. For example, the production of the fungal polyketide 6-methylsalicylic acid (6-MSA) in heterologous *E. coli*, yeast, and plant cells has been reported. See Kealey *et al.*, Jan. 1998, Production of a polyketide natural product in nonpolyketide-producing prokaryotic and eukaryotic host, *Proc. Natl. Acad. Sci. USA* 95:505-9, U.S. Patent No. 6,033,883, and PCT Patent Publication Nos. 98/27203 and 99/02669, each of which is incorporated herein by reference. Heterologous production of 6-MSA required or was considerably increased by co-expression of a heterologous acyl carrier protein synthase (ACPS) and that, for *E. coli*, media supplements were helpful in increasing the level of the malonyl CoA substrate utilized in 6-MSA biosynthesis. See also, PCT Patent Publication No. 97/13845, incorporated herein by reference.

The biosynthesis of other polyketides requires substrates other than or in addition to malonyl CoA. Such substrates include, for example, propionyl CoA, 2-methylmalonyl CoA, 2-hydroxymalonyl CoA, and 2-ethylmalonyl CoA. Of the myriad host cells possible for utilization as polyketide producing
5 hosts, many do not naturally produce such substrates. Given the potential for making valuable and useful polyketides in large quantities in heterologous host cells, there is a need for host cells capable of making the substrates required for polyketide biosynthesis. The present invention helps meet that need by providing recombinant host cells, expression vectors, and methods
10 for making polyketides in diverse host cells.

Summary of the Invention

The present invention provides recombinant host cells and expression vectors for making products in host cells that are otherwise unable to make
15 those products due to the lack of a biosynthetic pathway to produce a precursor required for biosynthesis of the product. The present invention also provides methods for increasing the amounts of a product produced in a host cell by providing recombinant biosynthetic pathways for production of a precursor utilized in the biosynthesis of a product.

20 In one embodiment, the host cell does not produce the precursor, and the host cell is modified by introduction of a recombinant expression vector so that it can produce the precursor. In another embodiment, the precursor is produced in the host cell in small amounts, and the host cell is modified by introduction of a recombinant expression vector so that it can produce the
25 precursor in larger amounts. In a preferred embodiment, the precursor is a primary metabolite that is produced in first cell but not in a second heterologous cell. In accordance with the methods of the invention, the genes that encode the enzymes that produce the primary metabolite in the first cell are transferred to the second cell. The transfer is accomplished using an
30 expression vector of the invention. The expression vector drives expression of the genes and production of the metabolite in the second cell.

In a preferred embodiment, the product is a polyketide. The polyketide is a polyketide synthesized by either a modular, iterative, or fungal PKS. The precursor is selected from the group consisting of malonyl CoA, propionyl CoA, methylmalonyl CoA, ethylmalonyl CoA, and hydroxymalonyl or methoxymalonyl CoA. In an especially preferred embodiment, the polyketide utilizes methylmalonyl CoA in its biosynthesis. In one preferred embodiment, the polyketide is synthesized by a modular PKS that requires methylmalonyl CoA to synthesize the polyketide.

In one embodiment, the host cell is either a procaryotic or eukaryotic host cell. In one embodiment, the host cell is an *E. coli* host cell. In another embodiment, the host cell is a yeast host cell. In another embodiment, the host cell is an Actinomycetes host cell, including but not limited to a *Streptomyces* host cell. In another embodiment, the host cell is a plant host cell. In a preferred embodiment, the host cell is either an *E. coli* or yeast host cell, the product is a polyketide, and the precursor is methylmalonyl CoA.

In one embodiment, the invention provides a recombinant expression vector that comprises a promoter positioned to drive expression of one or more genes that encode the enzymes required for biosynthesis of a precursor. In a preferred embodiment, the promoter is derived from a PKS gene. In a related embodiment, the invention provides recombinant host cells comprising one or more expression vectors that drive expression of the enzymes that produce the precursor.

In another embodiment, the invention provides a recombinant host cell that comprises not only an expression vector of the invention but also an expression vector that comprises a promoter positioned to drive expression of a PKS. In a related embodiment, the invention provides recombinant host cells comprising the vector that produces the PKS and its corresponding polyketide. In a preferred embodiment, the host cell is an *E. coli* or yeast host cell.

These and other embodiments of the invention are described in more detail in the following description, the examples, and claims set forth below.

Brief Description of the Figures

Figure 1 shows the modules and domains of DEBS and the biosynthesis of 6-dEB from propionyl CoA and methylmalonyl CoA.

5

Detailed Description of the Invention

The present invention provides recombinant host cells and expression vectors for making products in host cells, which are otherwise unable to make those products due to the lack of a biosynthetic pathway to produce a precursor required for biosynthesis of the product. As used herein, the term recombinant refers to a cell, compound, or composition produced at least in part by human intervention, particularly by modification of the genetic material. The present invention also provides methods for increasing the amounts of a product produced in a host cell by providing recombinant biosynthetic pathways for production of a precursor utilized in the biosynthesis of a product.

In one embodiment, the host cell does not produce the precursor, and the host cell is modified by introduction of a recombinant expression vector so that it can produce the precursor. In another embodiment, the precursor is produced in the host cell in small amounts, and the host cell is modified by introduction of a recombinant expression vector so that it can produce the precursor in larger amounts. In a preferred embodiment, the precursor is a primary metabolite that is produced in first cell but not in a second heterologous cell. In accordance with the methods of the invention, the genes that encode the enzymes that produce the primary metabolite in the first cell are transferred to the second cell. The transfer is accomplished using an expression vector of the invention. The expression vector drives expression of the genes and production of the metabolite in the second cell.

The invention, in its most general form, concerns the introduction, in whole or in part, of a metabolic pathway from one cell into a heterologous host cell. The invention also encompasses the modification of an existing

metabolic pathway, in whole or in part, in a cell, through the introduction of heterologous genetic material into the cell. In all embodiments, the resulting cell is different with regard to its cellular physiology and biochemistry in a manner such that the bio-synthesis, bio-degradation, transport, biochemical
5 modification, or levels of intracellular metabolites allow production or improve expression of desired products. The invention is exemplified by increasing the level of polyketides produced in a heterologous host and by restricting the chemical composition of products to the desired structures.

Thus, in a preferred embodiment, the product produced by the cell is a
10 polyketide. The polyketide is a polyketide synthesized by either a modular, iterative, or fungal PKS. The precursor is selected from the group consisting of malonyl CoA, propionyl CoA, methylmalonyl CoA, ethylmalonyl CoA, and hydroxymalonyl or methoxymalonyl CoA. In an especially preferred embodiment, the polyketide utilizes methylmalonyl CoA in its biosynthesis.
15 In one preferred embodiment, the polyketide is synthesized by a modular PKS that requires methylmalonyl CoA to synthesize the polyketide.

The polyketide class of natural products includes members having diverse structural and pharmacological properties (see Monaghan and Tkacz, 1990, *Annu. Rev. Microbiol.* 44: 271, incorporated herein by reference).
20 Polyketides are assembled by polyketide synthases through successive condensations of activated coenzyme-A thioester monomers derived from small organic acids such as acetate, propionate, and butyrate. Active sites required for condensation include an acyltransferase (AT), acyl carrier protein (ACP), and beta-ketoacylsynthase (KS). Each condensation cycle results in a
25 β -keto group that undergoes all, some, or none of a series of processing activities. Active sites that perform these reactions include a ketoreductase (KR), dehydratase (DH), and enoylreductase (ER). Thus, the absence of any beta-keto processing domain results in the presence of a ketone, a KR alone gives rise to a hydroxyl, a KR and DH result in an alkene, while a KR, DH,
30 and ER combination leads to complete reduction to an alkane. After assembly of the polyketide chain, the molecule typically undergoes cyclization(s) and

post-PKS modification (e.g. glycosylation, oxidation, acylation) to achieve the final active compound.

Macrolides such as erythromycin and megalomicin are synthesized by modular PKSs (see Cane *et al.*, 1998, *Science* 282: 63, incorporated herein by reference). For illustrative purposes, the PKS that produces the erythromycin polyketide (6-deoxyerythronolide B synthase or DEBS; see U.S. Patent No. 5,824,513, incorporated herein by reference) is shown in Figure 1. DEBS is the most characterized and extensively used modular PKS system. DEBS synthesizes the polyketide 6-deoxyerythronolide B (6-dEB) from propionyl CoA and methylmalonyl CoA. In modular PKS enzymes such as DEBS, the enzymatic steps for each round of condensation and reduction are encoded within a single "module" of the polypeptide (i.e., one distinct module for every condensation cycle). DEBS consists of a loading module and 6 extender modules and a chain terminating thioesterase (TE) domain within three extremely large polypeptides encoded by three open reading frames (ORFs, designated *eryAI*, *eryAII*, and *eryAIII*).

Each of the three polypeptide subunits of DEBS (DEBSI, DEBSII, and DEBSIII) contains 2 extender modules, DEBSI additionally contains the loading module. Collectively, these proteins catalyze the condensation and appropriate reduction of 1 propionyl CoA starter unit and 6 methylmalonyl CoA extender units. Modules 1, 2, 5, and 6 contain KR domains; module 4 contains a complete set, KR/DH/ER, of reductive and dehydratase domains; and module 3 contains no functional reductive domain. Following the condensation and appropriate dehydration and reduction reactions, the enzyme bound intermediate is lactonized by the TE at the end of extender module 6 to form 6-dEB.

More particularly, the loading module of DEBS consists of two domains, an acyl-transferase (AT) domain and an acyl carrier protein (ACP) domain. In other PKS enzymes, the loading module is not composed of an AT and an ACP but instead utilizes a partially inactivated KS, an AT, and an ACP. This partially inactivated KS is in most instances called KS^Q, where the

superscript letter is the abbreviation for the amino acid, glutamine, that is present instead of the active site cysteine required for full activity. The AT domain of the loading module recognizes a particular acyl CoA (propionyl for DEBS, which can also accept acetyl) and transfers it as a thiol ester to the ACP of the loading module. Concurrently, the AT on each of the extender modules recognizes a particular extender-CoA (methylmalonyl for DEBS) and transfers it to the ACP of that module to form a thioester. Once the PKS is primed with acyl- and malonyl-ACPs, the acyl group of the loading module migrates to form a thiol ester (trans-esterification) at the KS of the first extender module; at this stage, extender module 1 possesses an acyl-KS and a methylmalonyl ACP. The acyl group derived from the loading module is then covalently attached to the alpha-carbon of the malonyl group to form a carbon-carbon bond, driven by concomitant decarboxylation, and generating a new acyl-ACP that has a backbone two carbons longer than the loading unit (elongation or extension). The growing polyketide chain is transferred from the ACP to the KS of the next module, and the process continues.

The polyketide chain, growing by two carbons each module, is sequentially passed as a covalently bound thiol ester from module to module, in an assembly line-like process. The carbon chain produced by this process alone would possess a ketone at every other carbon atom, producing a polyketone, from which the name polyketide arises. Commonly, however, the beta keto group of each two-carbon unit is modified just after it has been added to the growing polyketide chain but before it is transferred to the next module by either a KR, a KR plus a DH, or a KR, a DH, and an ER. As noted above, modules may contain additional enzymatic activities as well.

Once a polyketide chain traverses the final extender module of a PKS, it encounters the releasing domain or thioesterase found at the carboxyl end of most PKSs. Here, the polyketide is cleaved from the enzyme and typically cyclized. The resulting polyketide can be modified further by tailoring or modification enzymes; these enzymes add carbohydrate groups or methyl groups, or make other modifications, i.e., oxidation or reduction, on the

polyketide core molecule. For example, the final steps in conversion of 6-dEB to erythromycin A include the actions of a number of modification enzymes, such as: C-6 hydroxylation, attachment of mycarose and desosamine sugars, C-12 hydroxylation (which produces erythromycin C), and conversion of mycarose to cladinose via O-methylation.

With this overview of PKS and post-PKS modification enzymes and their substrates, one can better appreciate the benefits provided by the present invention. DEBS is produced naturally in *Saccharopolyspora erythraea* and has been transferred to a variety of *Streptomyces* species, such as *S. coelicolor* CH999 and *S. lividans* K4-114 and K4-155, in which it functions without further modification of the host cell to produce 6-dEB. Thus, *S. erythraea*, *S. coelicolor*, and *S. lividans* make the required precursors for 6-dEB synthesis. However, many other non-*Saccharopolyspora*, non-*Streptomyces* host cells do not make all of the required precursors or make them only at levels sufficient to support only very small amounts of polyketide biosynthesis.

The present invention provides recombinant DNA expression vectors and methods for making a polyketide and its required precursors in any host cell. In one embodiment, the host cell is either a procaryotic or eukaryotic host cell. In a preferred embodiment, the host cell is an *E. coli* host cell. In another preferred embodiment, the host cell is a yeast host cell. In another embodiment, the host cell is a plant host cell. In a preferred embodiment, the host cell is either an *E. coli* or yeast host cell, the product is a polyketide, and the precursor is methylmalonyl CoA.

The recombinant expression vectors of the invention comprise a promoter positioned to drive expression of one or more genes that encode the enzymes required for biosynthesis of a precursor. In a preferred embodiment, the promoter is derived from a PKS gene. In another preferred embodiment, the promoter is one derived from a host cell gene or from a virus or phage that normally infects the host cell and is heterologous to the gene that encodes the biosynthetic enzyme.

In another embodiment, the invention provides a recombinant host cell that comprises not only an expression vector of the invention but also an expression vector that comprises a promoter positioned to drive expression of a PKS. In a related embodiment, the invention provides recombinant host
5 cells comprising the vector that produces the PKS and its corresponding polyketide. In a preferred embodiment, the host cell is an *E. coli* or yeast host cell.

Neither *E. coli* nor yeast makes sufficient methylmalonyl CoA to support biosynthesis of large amounts of polyketides that require
10 methylmalonyl CoA in their biosynthesis, and most species do not produce the methylmalonyl CoA substrate at all. In one embodiment, the present invention provides *E. coli*, yeast, and other host cells that produce methylmalonyl CoA in amounts sufficient to support polyketide biosynthesis. In preferred embodiments, the cells produce sufficient amounts of
15 methylmalonyl CoA to support biosynthesis of polyketides requiring methylmalonyl CoA for their biosynthesis at levels ranging from 1 µg/L, to 1 mg/L, to 10 mg/L, to 100 mg/L, to 1 g/L, to 10 g/L.

In one embodiment, the host cells of the invention have been modified to express a heterologous methylmalonyl CoA mutase. This enzyme, which
20 converts succinyl CoA to methylmalonyl CoA (although the reverse reaction is 20 times more favored) has been expressed in *E. coli* using a gene cloned from propionibacteria but was inactive due to the lack of vitamin B12. In accordance with the methods of the present invention, this enzyme can be made in an active form in *E. coli* and other host cells by either expressing
25 (constitutively or otherwise) a B12 transporter gene, such as the endogenous *E. coli* gene and/or by utilizing a media that facilitates B12 uptake (as used herein, B12 can refer to the precursor hydroxocobalamin, which is converted to B12). While certain methylmalonyl CoA mutases make the R-isomer, including the methylmalonyl CoA mutases derived from the
30 propionibacteria, the R-isomer can be converted to the S-isomer using an

epimerase. For example, epimerase genes from propionibacteria or *Streptomyces* can be employed for this purpose.

In another embodiment, the host cells of the invention have been modified to express a heterologous propionyl CoA carboxylase that converts
5 propionyl CoA to methylmalonyl CoA. In this embodiment, one can further increase the amount of methylmalonyl CoA precursor by culturing the cells in a media supplemented with propionate. In a preferred embodiment, the host cells are *E. coli* host cells.

Thus, in accordance with the methods of the invention, the
10 heterologous production of certain polyketides in *E. coli*, yeast, and other host organisms require both the heterologous expression of a desired PKS and also the enzymes that produce at least some of the substrate molecules required by the PKS. These substrate molecules, called precursors, are not normally
15 found as intracellular metabolites in the host organism or are present in low abundance. The present invention provides a method to produce or modify the composition or quantities of intracellular metabolites within a host organism where such metabolites are not naturally present or are present in non-optimal amounts.

A specific embodiment of the present invention concerns the
20 introduction and modification of biochemical pathways for methylmalonyl CoA biosynthesis. Methylmalonyl CoA, as noted above, is a substrate utilized for the synthesis of polyketides by many polyketide synthases. Some of the known biochemical pathways for the intracellular production of methylmalonyl CoA employ enzymes and their corresponding genes found in
25 certain organisms. These enzymes and genes have not been found, or are otherwise non-optimal, in other organisms. These other organisms include those that could otherwise be very useful as heterologous hosts for the production of polyketides. The present invention provides methods to engineer a host organism so that it contains a new or modified ability to
30 produce methylmalonyl CoA and/or to increase or decrease the levels of methylmalonyl CoA in the host.

As noted above, two biochemical pathways involving methylmalonyl CoA are particularly relevant to this aspect of the present invention. These pathways are the methylmalonyl CoA mutase pathway, hereafter referred to as the MUT pathway, and the propionyl CoA carboxylase pathway, hereafter referred to as the PCC pathway.

The MUT pathway includes the enzymes methylmalonyl CoA mutase (5.4.99.2, using the numbering system devised by the Nomenclature Committee of the International Union of biochemistry and Molecular Biology), methylmalonyl CoA epimerase (5.1.99.1), and malonyl CoA decarboxylase (4.1.1.9). The biochemical pathway includes the conversion of succinyl CoA to (R)-methylmalonyl CoA through the action of methylmalonyl CoA mutase (5.4.99.2) followed by the conversion of (R)-methylmalonyl CoA to (S)-methylmalonyl CoA through the action of methylmalonyl CoA epimerase (5.1.99.1). (S)-methylmalonyl CoA is a substrate utilized by several polyketide synthases. The enzyme malonyl CoA decarboxylase (4.1.1.9) catalyzes the decarboxylation of malonyl CoA but is also reported to catalyze the decarboxylation of (R)-methylmalonyl CoA to form propionyl CoA. Propionyl CoA is a substrate utilized by some polyketide synthases.

The PCC pathway includes the enzymes propionyl CoA carboxylase (6.4.1.3) and propionyl CoA synthetase (6.2.1.17). The biochemical pathway includes the conversion of propionate to propionyl CoA through the action of propionyl CoA synthetase (6.2.1.17) followed by the conversion of propionyl CoA to (S)-methylmalonyl CoA through the action of propionyl CoA carboxylase (6.4.1.3). (S)-methylmalonyl CoA is the substrate utilized by many polyketide synthases.

An illustrative embodiment of the present invention employs specific enzymes from these pathways. As those skilled in the art will recognize upon contemplation of this description of the invention, the invention can also be practiced using additional and/or alternative enzymes involved in the MUT and PCC pathways. Moreover, the invention can be practiced using

additional and alternative pathways for methylmalonyl CoA and other intracellular metabolites.

The methods of the invention involve the introduction of genetic material into a host strain of choice to modify or alter the cellular physiology and biochemistry of the host. Through the introduction of genetic material, the host strain acquires new properties, e.g. the ability to produce a new, or greater quantities of, an intracellular metabolite. In an illustrative embodiment of the invention, the introduction of genetic material into the host strain results in a new or modified ability to produce methylmalonyl CoA. The genetic material introduced into the host strain contains gene(s), or parts of genes, coding for one or more of the enzymes involved in the bio-synthesis/bio-degradation of methylmalonyl CoA and may also include additional elements for the expression and/or regulation of expression of these genes, e.g. promoter sequences. Specific gene sequences coding for enzymes involved in the bio-synthesis/bio-degradation of methylmalonyl CoA are listed below.

A suitable methylmalonyl CoA mutase (5.4.99.2) gene can be isolated from *Streptomyces cinnamonensis*. See Birch *et al.*, 1993, *J. Bacteriol.* 175:3511-3519, entitled "Cloning, sequencing, and expression of the gene encoding methylmalonyl-coenzyme A mutase from *Streptomyces cinnamonensis*." This enzyme is a two subunit enzyme; the A and B subunit coding sequences are available under Genbank accession L10064. Another suitable methylmalonyl CoA mutase gene can be isolated from *Propionibacterium shermanii*. See Marsh *et al.*, 1989, *Biochem. J.* 260: 345-352, entitled "Cloning and structural characterization of the genes coding for adenosylcobalamin-dependent methylmalonyl CoA mutase from *Propionibacterium shermanii*." Alternatively, a suitable methylmalonyl CoA mutase gene can be isolated from *Porphyromonas gingivalis*. See Jackson *et al.*, 1995, *Gene* 167: 127-132, entitled "Cloning, expression and sequence analysis of the genes encoding the heterodimeric methylmalonyl CoA mutase of *Porphyromonas gingivalis* W50." Alternatively, suitable methylmalonyl CoA mutase genes can be isolated from

any of the sources noted in the following table of a partial BLAST search report or from additional BLAST analyses.

Results of BLAST Search of NCBI Database for Methylmalonyl CoA

5 Mutase

mutA

- gb|L10064|STMMUTA Streptomyces cinnamomensis 931 0.0 (query sequence)
- gb|AD000015|MSGY175 Mycobacterium tuberculosis sequence 300 7e-80
- 10 emb|Z79701|MTCY277 Mycobacterium tuberculosis H37Rv 300 7e-80
- gb|AD000001|MSGY456 Mycobacterium tuberculosis sequence 238 8e-76
- emb|X14965|PSMUTAB Propionibacterium shermanii mutA 268 5e-70
- gb|L30136|POYMCMA B Porphyromonas gingivalis 137 9e-31
- gb|AE000375|AE000375 Escherichia coli K-12 MG1655 134 1e-29
- 15 gb|U28377|ECU28377 Escherichia coli K-12 genome; 134 1e-29
- emb|X66836|ECSERAICI E.coli serA, iciA, sbm genes 133 1e-29
- gb|AF080073|SMPCAS2 Sinorhizobium meliloti 130 2e-28
- ref|NM_000255.1|MUT| Homo sapiens 113 2e-23
- dbj|AP000006|AP000006 Pyrococcus horikoshii OT3 110 2e-22
- 20 emb|AJ248285.1|CNSPAX03 Pyrococcus abyssi 109 3e-22
- emb|X51941|MMMMCOAM Mouse mRNA 109 3e-22
- gb|AE000952|AE000952 Archaeoglobus fulgidus section 155 104 9e-21
- emb|AJ237976.1|SCO237976 Streptomyces coelicolor icmA gene 103 2e-20
- dbj|AP000062.1|AP000062 Aeropyrum pernix genomic DNA 102 3e-20
- 25 gb|U67612|SCU67612 Streptomyces cinnamomensis coenzyme B12 98 7e-19
- gb|AE001015|AE001015 Archaeoglobus fulgidus section 92 97 1e-18
- emb|X59424|BFOF4 Bacillus firmus OF4 genes for ATP binding 82 7e-14

mutB

- 30 gb|L10064|STMMUTA Streptomyces cinnamomensis 1379 0.0 (query sequence)
- gb|AD000001|MSGY456 Mycobacterium tuberculosis 1018 0.0
- emb|Z79701|MTCY277 Mycobacterium tuberculosis H37Rv 1017 0.0
- gb|AD000015|MSGY175 Mycobacterium tuberculosis sequence 1017 0.0
- 35 emb|X14965|PSMUTAB Propionibacterium shermanii 996 0.0
- gb|L30136|POYMCMA B Porphyromonas gingivalis methylmalonyl 882 0.0
- ref|NM_000255.1|MUT| Homo sapiens methylmalonyl Coenzyme A 855 0.0
- emb|X51941|MMMMCOAM Mouse mRNA 32 0.0
- 40 gb|U28377|ECU28377 Escherichia coli K-12 genome 798 0.0
- gb|AE000375|AE000375 Escherichia coli K-12 MG1655 798 0.0
- emb|X66836|ECSERAICI E.coli serA, iciA, sbm genes 797 0.0
- gb|AF080073|SMPCAS2 Sinorhizobium meliloti 782 0.0
- gb|AE001015|AE001015 Archaeoglobus fulgidus 516 e-145
- 45 dbj|AP000062.1|AP000062 Aeropyrum pernix genomic DNA 408 e-139

- emb|AJ248285.1|CNSPAX03 *Pyrococcus abyssi* complete genome 486 e-135
 dbj|AP000006|AP000006 *Pyrococcus horikoshii* OT3 genomic DNA 480 e-133
 gb|AE000952|AE000952 *Archaeoglobus fulgidus* section 155 467 e-130
 5 emb|Z35604.1|CEZK1058 *Caenorhabditis elegans* cosmid ZK1058 316 e-109
 emb|AJ237976.1|SCO237976 *Streptomyces coelicolor* icmA 377 e-103
 gb|U67612|SCU67612 *Streptomyces cinnamonensis* coenzyme 372 e-101
 emb|AL035161|SC9C7 *Streptomyces coelicolor* cosmid 9C7 359 2e-97
 gb|U28335|MEU28335 *Methylobacterium extorquens* 351 4e-95
 10 gb|AF008569|AF008569 *Streptomyces collinus* coenzyme 337 8e-91
 gb|U65074|ECU65074 *Escherichia coli* chromosome 275 3e-72
 gb|M37500|HUMMUT03 Human methylmalonyl CoA mutase 202 3e-50
 gb|AF178673.1|AF178673 *Streptomyces cinnamonensis* 183 1e-44
 emb|Z49936.1|CEF13B10 *Caenorhabditis elegans* cosmid F13B10 138 2e-41
 15 gb|M37499|HUMMUT02 Human methylmalonyl CoA mutase 112 4e-23
 dbj|AP000001.1|AP000001 *Pyrococcus horikoshii* OT3 genomic 106 2e-21
 emb|AJ248283.1|CNSPAX01 *Pyrococcus abyssi* complete genome 106 2e-21
 gb|M37503|HUMMUT06 Human methylmalonyl CoA mutase 101 7e-20
 gb|M37508|HUMMUT11 Human methylmalonyl CoA mutase 86 3e-15
 20 gb|M37509|HUMMUT12 Human methylmalonyl CoA mutase 80 3e-13
 gb|M37501|HUMMUT04 Human methylmalonyl CoA mutase 77 2e-12

Methylmalonyl CoA mutase requires vitamin B12 (adenosylcobalamin) as an essential cofactor for activity. One of the difficulties in expressing active
 25 methylmalonyl CoA mutase in a heterologous host is that the host organism may not provide sufficient, if any, amounts of this cofactor. Work on the expression of methionine synthase, a cobalamin-dependent enzyme, in *E. coli*, a host that does not synthesize cobalamin, has shown that it is possible to express an active cobalamin-dependent enzyme by increasing the rate of
 30 cobalamin transport. See Amaratunga *et al.*, 1996, *Biochemistry* 35: 2453-2463, entitled "A synthetic module for the *metH* gene permits facile mutagenesis of the cobalamin-binding region of *Escherichia coli* methionine synthase: initial characterization of seven mutant proteins," incorporated herein by reference.

The methods of the present invention include the step of increasing the
 35 availability of cobalamin for the heterologous expression of active methylmalonyl CoA mutase in certain hosts, e.g. *E. coli*. In particular, these methods incorporate growing cells in a media that contains hydroxocobalamin and/or other nutrients, as described in Amaratunga *et al.*,

supra. Additional methods for increasing the availability of cobalamin include constitutive and/or over-expression of vitamin B12 transporter proteins and/or their regulators.

A suitable methylmalonyl CoA epimerase (5.1.99.1) gene for purposes
5 of the present invention can be isolated from *Streptomyces coelicolor* as reported in GenBank locus SC5F2A as gene SC5F2A.13 (referred to here as EP5) or from *S. coelicolor* as reported in GenBank locus SC6A5 as gene SC6A5.34 (referred to here as EP6). See Redenbach *et al.*, 1996, *Mol. Microbiol.* 21 (1), 77-96, entitled "A set of ordered cosmids and a detailed genetic and
10 physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome," incorporated herein by reference. To date, no biochemical characterization of the proteins encoded by the genes EP5 and EP6 has been carried out; thus, the present invention provides a method for using these genes to provide methylmalonyl CoA epimerase activity to a host. That these genes encode
15 proteins with methylmalonyl CoA epimerase activity is supported by their homology to the sequence of a 2-arylpropionyl CoA epimerase from rat. See Reichel *et al.*, 1997, *Mol. Pharmacol.* 51: 576-582, entitled "Molecular cloning and expression of a 2-arylpropionyl-coenzyme A epimerase: a key enzyme in the inversion metabolism of ibuprofen," and Shieh & Chen, 1993, *J. Biol. Chem.* 268: 3487-3493, entitled "Purification and characterization of novel '2-
20 arylpropionyl CoA epimerases' from rat liver cytosol and mitochondria." Both rat 2-arylpropionyl CoA epimerase and methylmalonyl CoA epimerase catalyze the same stereoisomeric inversion, but with different chemical groups attached.

25 Biochemical characterization of a methylmalonyl CoA epimerase enzyme purified from *Propionibacterium shermanii* has been completed. See Leadlay, 1981, *Biochem. J.* 197: 413-419, entitled "Purification and characterization of methylmalonyl CoA epimerase from *Propionibacterium shermanii*," Leadlay & Fuller, 1983, *Biochem. J.* 213: 635-642, entitled "Proton
30 transfer in methylmalonyl CoA epimerase from *Propionibacterium shermanii*: Studies with specifically tritiated (2R)-methylmalonyl CoA as substrate; Fuller

& Leadlay, 1983, *Biochem. J.* 213: 643-650, entitled "Proton transfer in methylmalonyl CoA epimerase from *Propionibacterium shermanii*: The reaction of (2R)-methylmalonyl CoA in tritiated water." The DNA sequence of the gene coding for this enzyme from *Propionibacterium shermanii* is provided by the present invention in isolated and recombinant form and is incorporated into expression vectors and host cells of the invention. Suitable methylmalonyl CoA epimerase genes can be isolated from a BLAST search using the *P. shermanii* sequence provided in Example 1, below. Preferred epimerases in addition to the *P. shermanii* epimerase include gene identified by homology with the *P. shermanii* sequence located on cosmid 8F4 from the *S. coelicolor* genome sequencing project and the *B. subtilis* epimerase described by Haller *et al.*, 2000, *Biochemistry* 39 (16): 4622-4629, incorporated herein by reference.

One can also make S-methylmalonyl CoA from R-methylmalonyl CoA utilizing an activity of malonyl CoA decarboxylase A, which converts R-methylmalonyl CoA to propionyl CoA. As described above, propionyl CoA can then be converted to S-methylmalonyl CoA by propionyl CoA carboxylase. A suitable malonyl CoA decarboxylase (4.1.1.9) gene for purposes of the present invention can be isolated from *Saccharopolyspora erythraea* as reported in Hsieh & Kolattukudy, 1994, *J. Bacteriol.* 176: 714-724, entitled "Inhibition of erythromycin synthesis by disruption of malonyl-coenzyme A decarboxylase gene *eryM* in *Saccharopolyspora erythraea*." Alternatively, suitable malonyl CoA decarboxylase genes can be isolated from any of the sources noted in the following table of BLAST search reports or by additional BLAST searches.

Results of BLAST Search of NCBI Database for Malonyl CoA Decarboxylase

Malonyl CoA decarboxylase (DC)

gb|L05192|SERMALCOAD *S. erythraea* malonyl 664 0.0 (query sequence)
emb|AL022268|SC4H2 *Streptomyces coelicolor* cosmid 4H2 128 3e-28
emb|Z75555|MTCY02B10 *Mycobacterium tuberculosis* H37Rv 109 1e-22
gb|AD000018|MSGY151 *Mycobacterium tuberculosis* sequence 109 1e-22
gb|AF141323.1|AF141323 *Shigella flexneri* SHI-2 95 5e-18

- emb|X76100|ECIUC *E.coli* plasmid iucA, iucB and iucC genes 92 3e-17
 emb|AL116808.1|CNS01DGW *Botrytis cinerea* strain T4 cDNA 88 5e-16
 gb|AF110737.1|AF110737 *Sinorhizobium meliloti* strain 2011 84 9e-15
 emb|AL109846.1|SPBC17G9 *S.pombe* chromosome II cosmid c17G9 71
 5 7e-11
 gb|L06163|PSEAAC *Pseudomonas fluorescens* aminoglycoside 70 1e-10

A suitable propionyl CoA carboxylase (6.4.1.3) gene for purposes of the present invention can be isolated from *Streptomyces coelicolor* as reported in
 10 GenBank locus AF113605 (pccB), AF113604 (accA2) and AF113603 (accA1) by H. C. Gramajo and colleagues. The propionyl CoA carboxylase gene product requires biotin for activity. If the host cell does not make biotin, then the genes for biotin transport can be transferred to the host cell. Even if the host cell makes or transports biotin, the endogenous biotin transferase enzyme
 15 may not have sufficient activity (whether due to specificity constraints or other reasons) to biotinylate the propionyl CoA carboxylase at the rate required for high level precursor synthesis. In this event, one can simply provide the host cell with a sufficiently active biotin transferase enzyme gene, or if there is an endogenous transferase gene, such as the *birA* gene in *E. coli*,
 20 one can simply overexpress that gene by recombinant methods. Many additional genes coding for propionyl CoA carboxylases, or acetyl CoA carboxylases with relaxed substrate specificity that includes propionate, have been reported and can be used as sources for this gene, as shown in the following table.

25

Results of BLAST Search of NCBI Database for Propionyl CoA Carboxylase
Propionyl CoA Carboxylase (pccB)

- gb|AF113605.1|AF113605 *S. coelicolor* propionyl 1035 0.0 (query sequence)
 emb|X92557|SEPCCBBCP *S.erythraea* pccB, bcpA2, and orfX 800 0.0
 30 emb|Z92771|MTCY71 *Mycobacterium tuberculosis* H37Rv 691 0.0
 dbj|AB018531|AB018531 *Corynebacterium glutamicum* dtsR1 686 0.0
 gb|U00012|U00012 *Mycobacterium leprae* cosmid B1308 686 0.0
 dbj|AB018530|AB018530 *Corynebacterium glutamicum* dtsR gene 612 e-
 174
 35 gb|AE001742.1|AE001742 *Thermotoga maritima* section 54 610 e-173
 emb|AJ002015|PMAJ2015 *Propionigenium modestum* mmdD 589 e-167
 dbj|AB007000|AB007000 *Myxococcus xanthus* MxppcB gene 588 e-166

- gb|L48340|MTBKATA *Methylobacterium extorquens* catalase 588 e-166
 gb|AE000952|AE000952 *Archaeoglobus fulgidus* section 155 572 e-162
 dbj|AP000005|AP000005 *Pyrococcus horikoshii* OT3 genomic 570 e-161
 emb|AJ248285.1|CNSPAX03 *Pyrococcus abyssi* complete genome 570 e-161
 5 emb|AL031124|SC1C2 *Streptomyces coelicolor* cosmid 1C2 563 e-159
 gb|L22208|VEIMCDC *Veillonella parvula* methylmalonyl CoA 558 e-157
 gb|AF080235|AF080235 *Streptomyces cyanogenus* landomycin 552 e-155
 emb|AJ235272|RPXX03 *Rickettsia prowazekii* strain Madrid E 545 e-153
 dbj|AB000886|AB000886 *Sus scrofa* mRNA for Propionyl CoA 539 e-152
 10 ref|NM_000532.1|PCCB| *Homo sapiens* propionyl Coenzyme A 538 e-151
 emb|X73424|HSPCCBA *Homo sapiens* gene for propionyl CoA 538 e-151
 gb|M14634|RATPCCB Rat mitochondrial propionyl CoA 535 e-150
 gb|S67325|S67325 propionyl CoA carboxylase beta subunit 531 e-149
 gb|U56964|CELF52E4 *Caenorhabditis elegans* cosmid F52E4 367 e-143
 15 emb|Z99116|BSUB0013 *Bacillus subtilis* complete genome 494 e-138
 dbj|D84432|BACJH642 *Bacillus subtilis* DNA, 283 Kb region 494 e-138
 gb|AF042099|AF042099 *Sulfolobus metallicus* putative 486 e-136
 emb|AL022076.1|MTV026 *Mycobacterium tuberculosis* H37Rv 483 e-135
 gb|L04196|PRSTRANSC *Propionibacterium shermanii* 383 e-104
 20 emb|AL023635.1|MLCB1243 *Mycobacterium leprae* cosmid B1243 356 1e-96
 emb|Z70692.1|MTCY427 *Mycobacterium tuberculosis* H37Rv 353 1e-95
 gb|L78825|MSG1723CS *Mycobacterium leprae* cosmid B1723 DNA 319 4e-93
 25 gb|M95713|RERCOABETA *Rhodococcus erythropolis* 340 5e-92
 emb|Z99113|BSUB0010 *Bacillus subtilis* complete genome 325 2e-87
 gb|U94697|CCU94697 *Caulobacter crescentus* DNA topoisomerase 270 6e-71
 emb|Z95556|MTCY07A7 *Mycobacterium tuberculosis* H37Rv 253 9e-66
 30 emb|Y07660|MTACCBC *M. tuberculosis* accBC gene 231 6e-59
 emb|Z79700|MTCY10D7 *Mycobacterium tuberculosis* H37Rv 229 2e-58
 dbj|AB018557.1|AB018557 *Streptomyces griseus* cyaA gene 228 5e-58
 gb|U46844|MSU46844 *Mycobacterium smegmatis* catalase 209 2e-52
 emb|Z19555.1|CEF02A9 *Caenorhabditis elegans* cosmid F02A9 105 9e-51
 35 gb|M13573|HUMPCCB Human propionyl CoA carboxylase beta 194 5e-48
 gb|AF030576|AF030576 *Acidaminococcus fermentans* 170 9e-41
 emb|Y13917|BSY13917 *Bacillus subtilis* ppsE, yngL, yngK 149 2e-34
 emb|X69435|AFGCDA *A. fermentans* GCDA gene for 107 1e-21
 40 emb|Z82368|RPZ82368 *R. prowazekii* genomic DNA fragment 93 2e-17
 gb|AF025469|CEW09B6 *Caenorhabditis elegans* cosmid W09B6 78 5e-13
 gb|U87980|MRU87980 *Malonomonas rubra* putative IS-element 78 7e-13
 gb|AE001518|AE001518 *Helicobacter pylori*, strain J99 75 6e-12
 45 gb|AE000604.1|AE000604 *Helicobacter pylori* 26695 section 82 75 8e-12
 gb|U89347|ACU89347 *Acinetobacter calcoaceticus* malonate 74 1e-11
 emb|AL021961|ATF28A23 *Arabidopsis thaliana* DNA 61 2e-11

- gb|AE001591|AE001591 *Chlamydia pneumoniae* section 7 73 2e-11
 emb|Z46886|UMACCGEN *U. maydis* ACC gene for acetyl coa 71 1e-10
 gb|U86128|SSPCCB1 *Sus scrofa* propionyl CoA carboxylase B 70 2e-10
 emb|AJ006497|HSA006497 *Homo sapiens* PCCB gene, exons 11 70 2e-10
 5 gb|AE001301|AE001301 *Chlamydia trachomatis* section 28 69 5e-10
 gb|U32724|U32724 *Haemophilus influenzae* Rd section 39 68 8e-10
 gb|U04358|PSU04358 *Pseudomonas syringae* pv. *syringae* Y30 68 8e-10

Propionyl CoA carboxylase (accA2)

- 10 gb|AF113604.1|AF113604 *S. coelicolor* putative 1101 0.0 (query sequence)
 gb|AF113603.1|AF113603 *Streptomyces coelicolor* putative 1090 0.0
 gb|AF126429.1|AF126429 *Streptomyces venezuelae* JadJ 967 0.0
 emb|Z92771|MTCY71 *Mycobacterium tuberculosis* H37Rv 758 0.0
 emb|X92557|SEPCCBSCP *S. erythraea* pccB, bcpA2, and orfX genes 753
 15 0.0
 emb|X92556|SEHGATABCP *S. erythraea* hgtA, bcpA1, and orf122 753 0.0
 gb|U00012|U00012 *Mycobacterium leprae* cosmid B1308 746 0.0
 emb|X63470|MLBCCPG *M. leprae* gene for biotin carboxyl 743 0.0
 gb|U35023|CGU35023 *Corynebacterium glutamicum* thiosulfate 695 0.0
 20 gb|U24659|SVU24659 *Streptomyces venezuelae* glucose 599 e-170
 gb|AE000742|AE000742 *Aquifex aeolicus* section 74 413 e-113
 gb|U67563|U67563 *Methanococcus jannaschii* section 105 405 e-111
 gb|L36530|MQSPYRCARB *Aedes aegypti* pyruvate carboxylase 400 e-110
 gb|AF132152.1|AF132152 *Drosophila melanogaster* clone 396 e-108
 25 gb|L09192|MUSMPYR *Mus musculus* pyruvate carboxylase 393 e-107
 gb|U36585|RNU36585 *Rattus norvegicus* pyruvate carboxylase 391 e-107
 gb|U32314|RNU32314 *Rattus norvegicus* pyruvate carboxylase 391 e-107
 gb|L14862|ANAACCC *Anabaena* sp. (PCC 7120) 49.1 kDa biotin 388 e-106
 gb|U59234|SPU59234 *Synechococcus* PCC7942 biotin 387 e-106
 30 gb|U04641|HSU04641 Human pyruvate carboxylase (PC) mRNA 387 e-106
 ref|NM_000920.1|PC| *Homo sapiens* pyruvate carboxylase (PC) 386 e-105
 gb|AE001090|AE001090 *Archaeoglobus fulgidus* section 17 383 e-104
 dbj|D84432|BACJH642 *Bacillus subtilis* DNA, 283 Kb region 382 e-104
 emb|Z99116|BSUB0013 *Bacillus subtilis* complete genome 382 e-104
 35 gb|AE000942|AE000942 *Methanobacterium thermoautotrophicum* 382 e-104
 gb|S72370|S72370 pyruvate carboxylase human, kidney 380 e-104
 dbj|D64001|SYCCPNC *Synechocystis* sp. PCC6803 complete 379 e-103
 gb|L14612|PSEACCBBC *Pseudomonas aeruginosa* biotin carboxyl 376 e-103
 40 gb|U32778|U32778 *Haemophilus influenzae* Rd section 93 375 e-102
 emb|Z36087|SCYBR218C *S. cerevisiae* chromosome II 374 e-102
 gb|U35647|SCU35647 *Saccharomyces cerevisiae* pyruvate 374 e-102
 gb|J03889|YSCPCB Yeast (*S. cerevisiae*) pyruvate carboxylase 374 e-102
 gb|U90879|ATU90879 *Arabidopsis thaliana* biotin carboxylase 374 e-102
 45 emb|Z72584|SCYGL062W *S. cerevisiae* chromosome VII 374 e-102
 emb|X59890|SCPYC2G *S. cerevisiae* PYC2 gene for pyruvate 373 e-102

- gb|AE000749|AE000749 Aquifex aeolicus section 81 371 e-101
 gb|AE001286|AE001286 Chlamydia trachomatis section 13 370 e-101
 gb|AE001604|AE001604 Chlamydia pneumoniae section 20 369 e-100
 gb|AF007100|AF007100 Glycine max biotin carboxylase 368 e-100
 5 emb|Z95556|MTCY07A7 Mycobacterium tuberculosis H37Rv 367 e-100
 emb|Z19549|MTBCARBCP M.tuberculosis gene for biotin 367 e-100
 gb|AF068249|AF068249 Glycine max biotin carboxylase 366 1e-99
 gb|L38260|TOBBCSO Nicotiana tabacum acetyl CoA 363 7e-99
 gb|U36245|BSU36245 Bacillus subtilis biotin carboxyl 362 2e-98
 10 gb|AF097728|AF097728 Aspergillus terreus pyruvate 361 3e-98
 emb|AJ235272|RPXX03 Rickettsia prowazekii strain Madrid E 360 1e-97
 dbj|D83706|D83706 Bacillus stearothermophilus DNA 360 1e-97
 gb|AE000744|AE000744 Aquifex aeolicus section 76 358 3e-97
 emb|AL109846.1|SPBC17G9 S.pombe chromosome II 356 1e-96
 15 dbj|D78170|D78170 Yeast DNA for pyruvate carboxylase 353 1e-95
 gb|M79446|ECOFABG Escherichia coli biotin carboxylase gene 352 2e-95
 gb|M83198|ECOFABEGF Escherichia coli biotin carboxyl 352 2e-95
 gb|AE000404|AE000404 Escherichia coli K-12 MG1655 352 2e-95
 gb|U18997.1|ECOUW67 Escherichia coli K-12 chromosomal 352 2e-95
 20 gb|M80458|ECOACOAC E.coli biotin carboxylase and biotin 352 2e-95
 gb|U51439|REU51439 Rhizobium etli pyruvate carboxylase 351 5e-95
 emb|Y13917|BSY13917 Bacillus subtilis ppsE, yngL, yngK 348 3e-94
 emb|Z99113|BSUB0010 Bacillus subtilis complete genome 348 3e-94
 gb|AE001274.1|AE001274 Leishmania major chromosome 1 347 6e-94
 25 gb|AF042099|AF042099 Sulfolobus metallicus putative 346 1e-93
 emb|Z81052.1|CED2023 Caenorhabditis elegans cosmid D2023 162 3e-92
 emb|Z79700|MTCY10D7 Mycobacterium tuberculosis H37Rv 341 4e-92
 emb|Z99111|BSUB0008 Bacillus subtilis complete genome 340 1e-91
 gb|U12536|ATU12536 Arabidopsis thaliana 3-methylcrotonyl 338 4e-91
 30 emb|Y11106|PPPYC1 P.pastoris PYC1 gene 338 4e-91
 gb|AE001529|AE001529 Helicobacter pylori, strain J99 334 5e-90
 gb|AE000553.1|AE000553 Helicobacter pylori 26695333 7e-90
 emb|Y09548|CGPYC Corynebacterium glutamicum pyc gene 333 1e-89
 35 gb|AF038548|AF038548 Corynebacterium glutamicum pyruvate 333 1e-89
 ref|NM_000282.1|PCCA Homo sapiens Propionyl Coenzyme 333 1e-89
 gb|M22631|RATPCOA Rat alpha-propionyl CoA carboxylase 332 2e-89
 gb|U08469|GMU08469 Glycine max 3-methylcrotonyl CoA 328 3e-88
 emb|Z83018|MTCY349 Mycobacterium tuberculosis H37Rv 318 4e-85
 40 emb|AJ243652.1|PFL243652 Pseudomonas fluorescens uahA gene 316 1e-84
 emb|Z36077|SCYBR208C S.cerevisiae chromosome II 312 2e-83
 gb|M64926|YSCUAMD Yeast urea amidolyase (DUR1.2) gene 311 5e-83
 emb|Z97025|BSZ97025 Bacillus subtilis nprE, yla[A,B,C,D,E,F 300 1e-79
 emb|Z81074.1|CEF32B6 Caenorhabditis elegans cosmid F32B6 131 7e-78
 45 gb|U00024|MTU00024 Mycobacterium tuberculosis cosmid tbc2 284 7e-75
 gb|AD000009|MSGY2 Mycobacterium tuberculosis sequence 284 7e-75

- gb|U34393|GMU34393 Glycine max acetyl CoA carboxylase 259 2e-67
 gb|U49829|CELF27D9 Caenorhabditis elegans cosmid F27D9 186 4e-59
 emb|AJ010111.1|BCE010111 Bacillus cereus pycA, ctaA, ctaB 208 5e-52
 gb|U19183|ZMU19183 Zea mays acetyl-coenzyme A carboxylase 208 5e-52
 5 gb|U10187|TAU10187 Triticum aestivum Tam 107 206 2e-51
 gb|AF029895|AF029895 Triticum aestivum acetyl-coenzyme A 205 5e-51
 gb|J03808|RATACACA Rat acetyl-coenzyme A carboxylase mRNA 204 8e-51
 emb|X80045|OAACOAC O.aries mRNA for acetyl CoA carboxylase 203 1e-50
 10 emb|X68968|HSACOAC H.sapiens mRNA for acetyl CoA 203 2e-50
 emb|AJ132890.1|BTA132890 Bos taurus mRNA for acetyl 202 2e-50
 gb|J03541|CHKCOACA Chicken acetyl CoA carboxylase mRNA 202 3e-50
 dbj|D34630|ATHACCRNA Arabidopsis thaliana mRNA 199 2e-49
 15 gb|L25042|ALFACCASE Medicago sativa acetyl CoA carboxylase 198 5e-49
 emb|Z71631|SCYNR016C S.cerevisiae chromosome XIV 193 2e-47
 gb|M92156|YSCFAS3A Saccharomyces cerevisiae acetyl CoA 193 2e-47
 emb|Z49809|SC8261X S.cerevisiae chromosome XIII cosmid 8261 192 3e-47
 20 emb|Z22558|SCHFA1GN S.cerevisiae HFA1 gene 192 3e-47
 dbj|D78165|D78165 Saccharomyces cerevisiae DNA 192 3e-47
 emb|Z46886|UMACCGEN U.maydis ACC gene for acetyl coa 190 1e-46
 ref|NM_001093.1|ACACB| Homo sapiens acetyl Coenzyme A 181 5e-44

- 25 **Propionyl CoA carboxylase (accA1)**
 gb|AF113603.1|AF113603 S. coelicolor putative 1101 0.0 (query sequence)
 gb|AF113604.1|AF113604 Streptomyces coelicolor putative 1090 0.0
 gb|AF126429.1|AF126429 Streptomyces venezuelae JadJ (jadJ) 967 0.0
 emb|Z92771|MTCY71 Mycobacterium tuberculosis H37Rv 758 0.0
 30 emb|X92557|SEPCCBBP S.erythraea pccB, bcpA2, and orfX genes 753 0.0
 emb|X92556|SEHGATABCP S.erythraea hgtA, bcpA1, and orf122 753 0.0
 gb|U00012|U00012 Mycobacterium leprae cosmid B1308 745 0.0
 emb|X63470|MLBCCPG M.leprae gene for biotin carboxyl 742 0.0
 35 gb|U35023|CGU35023 Corynebacterium glutamicum thiosulfate 694 0.0
 gb|U24659|SVU24659 Streptomyces venezuelae glucose 596 e-169
 gb|AE000742|AE000742 Aquifex aeolicus section 74 417 e-115
 gb|U67563|U67563 Methanococcus jannaschii section 105 413 e-114
 gb|L36530|MQSPYRCARB Aedes aegypti pyruvate carboxylase 404 e-111
 40 gb|AF132152.1|AF132152 Drosophila melanogaster clone 400 e-110
 gb|L09192|MUSMPYR Mus musculus pyruvate carboxylase 397 e-109
 gb|U36585|RNU36585 Rattus norvegicus pyruvate carboxylase 395 e-108
 gb|U32314|RNU32314 Rattus norvegicus pyruvate carboxylase 395 e-108
 gb|L14862|ANAACCC Anabaena sp. (PCC 7120) 49.1 kDa biotin 394 e-108
 45 gb|U04641|HSU04641 Human pyruvate carboxylase (PC) mRNA 391 e-107

- gb|U59234|SPU59234 *Synechococcus* PCC7942 biotin carboxylase 391 e-107
 ref|NM_000920.1|PC| *Homo sapiens* pyruvate carboxylase (PC) 390 e-107
 gb|AE001090|AE001090 *Archaeoglobus fulgidus* section 17 389 e-106
 5 gb|AE000942|AE000942 *Methanobacterium thermoautotrophicum* 386 e-105
 gb|S72370|S72370 pyruvate carboxylase human, kidney 384 e-105
 dbj|D84432|BACJH642 *Bacillus subtilis* DNA, 283 Kb region 383 e-105
 emb|Z99116|BSUB0013 *Bacillus subtilis* complete genome 383 e-105
 10 dbj|D64001|SYCCPNC *Synechocystis* sp. PCC6803 383 e-104
 gb|U35647|SCU35647 *Saccharomyces cerevisiae* pyruvate 382 e-104
 emb|Z36087|SCYBR218C *S.cerevisiae* chromosome II 382 e-104
 emb|Z72584|SCYGL062W *S.cerevisiae* chromosome VII 381 e-104
 gb|J03889|YSCPCB Yeast (*S.cerevisiae*) pyruvate carboxylase 381 e-104
 15 gb|L14612|PSEACCBBC *Pseudomonas aeruginosa* biotin carboxyl 381 e-104
 emb|X59890|SCPYC2G *S.cerivisiae* PYC2 gene for pyruvate 381 e-104
 gb|U32778|U32778 *Haemophilus influenzae* Rd section 93 380 e-104
 gb|U90879|ATU90879 *Arabidopsis thaliana* biotin carboxylase 377 e-103
 gb|AE000749|AE000749 *Aquifex aeolicus* section 81 of 109 377 e-103
 20 gb|AE001286|AE001286 *Chlamydia trachomatis* section 13 375 e-102
 gb|AE001604|AE001604 *Chlamydia pneumoniae* section 20 374 e-102
 gb|AF007100|AF007100 *Glycine max* biotin carboxylase 372 e-101
 emb|Z95556|MTCY07A7 *Mycobacterium tuberculosis* H37Rv 369 e-100
 emb|Z19549|MTBCARBCP *M.tuberculosis* gene for biotin 369 e-100
 25 gb|AF068249|AF068249 *Glycine max* biotin carboxylase 369 e-100
 gb|L38260|TOBBCSO *Nicotiana tabacum* acetyl CoA 367 e-100
 gb|AF097728|AF097728 *Aspergillus terreus* pyruvate 366 1e-99
 gb|AE000744|AE000744 *Aquifex aeolicus* section 76 of 109 364 4e-99
 dbj|D83706|D83706 *Bacillus stearothermophilus* DNA 363 7e-99
 30 gb|U36245|BSU36245 *Bacillus subtilis* biotin carboxyl 363 7e-99
 emb|AL109846.1|SPBC17G9 *S.pombe* chromosome II 362 2e-98
 emb|AJ235272|RPXX03 *Rickettsia prowazekii* strain Madrid E 361 3e-98
 dbj|D78170|D78170 Yeast DNA for pyruvate carboxylase 359 2e-97
 gb|M80458|ECOACOAC *E.coli* biotin carboxylase and biotin 358 3e-97
 35 gb|M79446|ECOFABG *Escherichia coli* biotin carboxylase gene 358 3e-97
 gb|M83198|ECOFABEGF *Escherichia coli* biotin carboxyl 358 3e-97
 gb|AE000404|AE000404 *Escherichia coli* K-12 MG1655 358 3e-97
 gb|U18997.1|ECOUW67 *Escherichia coli* K-12 chromosomal 358 3e-97
 gb|U51439|REU51439 *Rhizobium etli* pyruvate carboxylase 355 3e-96
 40 emb|Y13917|BSY13917 *Bacillus subtilis* ppsE, yngL, yngK, 354 4e-96
 emb|Z99113|BSUB0010 *Bacillus subtilis* complete genome 354 4e-96
 gb|AE001274.1|AE001274 *Leishmania major* chromosome 1 351 3e-95
 gb|AF042099|AF042099 *Sulfolobus metallicus* putative 350 9e-95
 emb|Z79700|MTCY10D7 *Mycobacterium tuberculosis* H37Rv 347 6e-94
 45 emb|Z81052.1|CED2023 *Caenorhabditis elegans* cosmid D2023 168 1e-93
 emb|Y11106|PPPYC1 *P.pastoris* PYC1 gene 345 2e-93
 emb|Z99111|BSUB0008 *Bacillus subtilis* complete genome 343 8e-93

- ref|NM_000282.1|PCCA| Homo sapiens Propionyl Coenzyme 340 6e-92
 gb|M22631|RATPCOA Rat alpha-propionyl CoA carboxylase 340 1e-91
 gb|U12536|ATU12536 Arabidopsis thaliana 3-methylcrotonyl 339 2e-91
 emb|Y09548|CGPYC Corynebacterium glutamicum pyc gene 338 4e-91
- 5 gb|AF038548|AF038548 Corynebacterium glutamicum pyruvate 338 4e-91
 gb|AE001529|AE001529 Helicobacter pylori, strain J99 337 8e-91
 gb|AE000553.1|AE000553 Helicobacter pylori 26695 336 1e-90
 gb|U08469|GMU08469 Glycine max 3-methylcrotonyl CoA 329 2e-88
- 10 emb|AJ243652.1|PFL243652 Pseudomonas fluorescens uahA gene 323 1e-86
 emb|Z83018|MTCY349 Mycobacterium tuberculosis H37Rv 321 3e-86
 emb|Z36077|SCYBR208C S.cerevisiae chromosome II 314 5e-84
 gb|M64926|YSCUAMD Yeast urea amidolyase (DUR1.2) gene 312 2e-83
 emb|Z97025|BSZ97025 Bacillus subtilis nprE, yla[A,B,C,D,E, 303 1e-80
- 15 emb|Z81074.1|CEF32B6 Caenorhabditis elegans cosmid F32B6 130 1e-78
 gb|U00024|MTU00024 Mycobacterium tuberculosis cosmid tbc2 287 6e-76
 gb|AD000009|MSGY2 Mycobacterium tuberculosis sequence 287 6e-76
 gb|U34393|GMU34393 Glycine max acetyl CoA carboxylase 262 3e-68
- 20 gb|U49829|CELF27D9 Caenorhabditis elegans cosmid F27D9 190 2e-61
 gb|U10187|TAU10187 Triticum aestivum Tam 107 213 2e-53
 gb|U19183|ZMU19183 Zea mays acetyl-coenzyme A carboxylase 212 3e-53
 emb|AJ010111.1|BCE010111 Bacillus cereus pycA, ctaA, ctaB 212 4e-53
 gb|AF029895|AF029895 Triticum aestivum acetyl-coenzyme 209 2e-52
- 25 gb|J03808|RATACACA Rat acetyl-coenzyme A carboxylase 205 4e-51
 emb|X80045|OAACOAC O.aries mRNA for acetyl CoA 205 5e-51
 emb|X68968|HSACOAC H.sapiens mRNA for acetyl CoA 204 8e-51
 dbj|D34630|ATHACCRNA Arabidopsis thaliana mRNA 203 1e-50
 emb|AJ132890.1|BTA132890 Bos taurus mRNA for acetyl CoA 203 1e-50
- 30 gb|J03541|CHKCOACA Chicken acetyl CoA carboxylase mRNA 203 1e-50
 gb|L25042|ALFACCASE Medicago sativa acetyl CoA carboxylase 202 2e-50
 emb|Z71631|SCYNR016C S.cerevisiae chromosome XIV 196 1e-48
 gb|M92156|YSCFAS3A Saccharomyces cerevisiae acetyl CoA 196 1e-48
- 35 emb|Z49809|SC8261X S.cerevisiae chromosome XIII cosmid 8261 195 4e-48
 emb|Z22558|SCHFA1GN S.cerevisiae HFA1 gene 195 4e-48
 dbj|D78165|D78165 Saccharomyces cerevisiae DNA 195 4e-48
 emb|Z46886|UMACCGEN U.maydis ACC gene for acetyl coa 188 5e-46
- 40 gb|L20784|CCXACOAC Cyclotella cryptica acetyl CoA 182 2e-44

Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA compounds differing in their nucleotide sequences can be used to encode a given amino acid sequence of

45 the invention. The native DNA sequence encoding the biosynthetic enzymes

in the tables above are referenced herein merely to illustrate a preferred embodiment of the invention, and the invention includes DNA compounds of any sequence that encode the amino acid sequences of the polypeptides and proteins of the enzymes utilized in the methods of the invention. In similar
5 fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity. The present invention includes such polypeptides with alternate amino acid sequences, and the amino acid sequences encoded by the DNA sequences shown herein merely illustrate
10 preferred embodiments of the invention.

Thus, in an especially preferred embodiment, the present invention provides DNA molecules in the form of recombinant DNA expression vectors or plasmids, as described in more detail below, that encode one or more precursor biosynthetic enzymes. Generally, such vectors can either replicate
15 in the cytoplasm of the host cell or integrate into the chromosomal DNA of the host cell. In either case, the vector can be a stable vector (i.e., the vector remains present over many cell divisions, even if only with selective pressure) or a transient vector (i.e., the vector is gradually lost by host cells with increasing numbers of cell divisions). The invention provides DNA molecules
20 in isolated (i.e., not pure, but existing in a preparation in an abundance and/or concentration not found in nature) and purified (i.e., substantially free of contaminating materials or substantially free of materials with which the corresponding DNA would be found in nature) form.

In one important embodiment, the invention provides methods for the
25 heterologous expression of one or more of the biosynthetic genes involved in S-methylmalonyl CoA biosynthesis and recombinant DNA expression vectors useful in the method. Thus, included within the scope of the invention are recombinant expression vectors that include such nucleic acids. The term expression vector refers to a nucleic acid that can be introduced into a host
30 cell or cell-free transcription and translation system. An expression vector can be maintained permanently or transiently in a cell, whether as part of the

chromosomal or other DNA in the cell or in any cellular compartment, such as a replicating vector in the cytoplasm. An expression vector also comprises a promoter that drives expression of an RNA, which typically is translated into a polypeptide in the cell or cell extract. For efficient translation of RNA
5 into protein, the expression vector also typically contains a ribosome-binding site sequence positioned upstream of the start codon of the coding sequence of the gene to be expressed. Other elements, such as enhancers, secretion signal sequences, transcription termination sequences, and one or more marker genes by which host cells containing the vector can be identified
10 and/or selected, may also be present in an expression vector. Selectable markers, i.e., genes that confer antibiotic resistance or sensitivity, are preferred and confer a selectable phenotype on transformed cells when the cells are grown in an appropriate selective medium.

The various components of an expression vector can vary widely,
15 depending on the intended use of the vector and the host cell(s) in which the vector is intended to replicate or drive expression. Expression vector components suitable for the expression of genes and maintenance of vectors in *E. coli*, yeast, *Streptomyces*, and other commonly used cells are widely known and commercially available. For example, suitable promoters for
20 inclusion in the expression vectors of the invention include those that function in eucaryotic or procaryotic host cells. Promoters can comprise regulatory sequences that allow for regulation of expression relative to the growth of the host cell or that cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus. For *E. coli* and certain other
25 bacterial host cells, promoters derived from genes for biosynthetic enzymes, antibiotic-resistance conferring enzymes, and phage proteins can be used and include, for example, the galactose, lactose (*lac*), maltose, tryptophan (*trp*), beta-lactamase (*bla*), bacteriophage lambda PL, and T5 promoters. In addition, synthetic promoters, such as the *tac* promoter (U.S. Patent
30 No. 4,551,433), can also be used. For *E. coli* expression vectors, it is useful to include an *E. coli* origin of replication, such as from pUC, p1P, p1I, and pBR.

Thus, recombinant expression vectors contain at least one expression system, which, in turn, is composed of at least a portion of PKS and/or other biosynthetic gene coding sequences operably linked to a promoter and optionally termination sequences that operate to effect expression of the coding sequence in compatible host cells. The host cells are modified by transformation with the recombinant DNA expression vectors of the invention to contain the expression system sequences either as extrachromosomal elements or integrated into the chromosome. The resulting host cells of the invention are useful in methods to produce PKS enzymes as well as polyketides and antibiotics and other useful compounds derived therefrom.

Preferred host cells for purposes of selecting vector components for expression vectors of the present invention include fungal host cells such as yeast and procaryotic host cells such as *E. coli*, but mammalian host cells can also be used. In hosts such as yeasts, plants, or mammalian cells that ordinarily do not produce polyketides, it may be necessary to provide, also typically by recombinant means, suitable holo-ACP synthases to convert the recombinantly produced PKS to functionality. Provision of such enzymes is described, for example, in PCT publication Nos. WO 97/13845 and 98/27203, each of which is incorporated herein by reference.

The recombinant host cells of the invention can express all of the polyketide biosynthetic genes or only a subset of the same. For example, if only the genes for a PKS are expressed in a host cell that otherwise does not produce polyketide modifying enzymes (such as hydroxylation, epoxidation, or glycosylation enzymes) that can act on the polyketide produced, then the host cell produces unmodified polyketides, called macrolide aglycones. Such macrolide aglycones can be hydroxylated and glycosylated by adding them to the fermentation of a strain such as, for example, *Streptomyces antibioticus* or *Saccharopolyspora erythraea*, that contains the requisite modification enzymes.

There are a wide variety of diverse organisms that can modify macrolide aglycones to provide compounds with, or that can be readily

modified to have, useful activities. For example, *Saccharopolyspora erythraea* can convert 6-dEB to a variety of useful compounds. The erythronolide 6-dEB is converted by the *eryF* gene product to erythronolide B, which is, in turn, glycosylated by the *eryB* gene product to obtain 3-O-mycarosylerythronolide B, which contains L-mycarose at C-3. The enzyme *eryC* gene product then converts this compound to erythromycin D by glycosylation with D-desosamine at C-5. Erythromycin D, therefore, differs from 6-dEB through glycosylation and by the addition of a hydroxyl group at C-6. Erythromycin D can be converted to erythromycin B in a reaction catalyzed by the *eryG* gene product by methylating the L-mycarose residue at C-3. Erythromycin D is converted to erythromycin C by the addition of a hydroxyl group at C-12 in a reaction catalyzed by the *eryK* gene product. Erythromycin A is obtained from erythromycin C by methylation of the mycarose residue in a reaction catalyzed by the *eryG* gene product. The unmodified polyketides provided by the present invention, such as, for example, 6-dEB produced in *E. coli*, can be provided to cultures of *S. erythraea* and converted to the corresponding derivatives of erythromycins A, B, C, and D in accordance with the procedure provided in the examples below. To ensure that only the desired compound is produced, one can use an *S. erythraea eryA* mutant that is unable to produce 6-dEB but can still carry out the desired conversions (Weber *et al.*, 1985, *J. Bacteriol.* 164(1): 425-433). Also, one can employ other mutant strains, such as *eryB*, *eryC*, *eryG*, and/or *eryK* mutants, or mutant strains having mutations in multiple genes, to accumulate a preferred compound. The conversion can also be carried out in large fermentors for commercial production.

Moreover, there are other useful organisms that can be employed to hydroxylate and/or glycosylate the compounds of the invention. As described above, the organisms can be mutants unable to produce the polyketide normally produced in that organism, the fermentation can be carried out on plates or in large fermentors, and the compounds produced can be chemically altered after fermentation. Thus, *Streptomyces venezuelae*, which produces picromycin, contains enzymes that can transfer a desosaminyl

group to the C-5 hydroxyl and a hydroxyl group to the C-12 position. In addition, *S. venezuelae* contains a glucosylation activity that glucosylates the 2'-hydroxyl group of the desosamine sugar. This latter modification reduces antibiotic activity, but the glucosyl residue is removed by enzymatic action
5 prior to release of the polyketide from the cell. Another organism, *S. narbonensis*, contains the same modification enzymes as *S. venezuelae*, except the C-12 hydroxylase. Thus, the present invention provides the compounds produced by hydroxylation and glycosylation of the macrolide aglycones of the invention by action of the enzymes endogenous to *S. narbonensis* and *S.*
10 *venezuelae*.

Other organisms suitable for making compounds of the invention include *Micromonospora megalomicea*, *Streptomyces antibioticus*, *S. fradiae*, and *S. thermotolerans*. *M. megalomicea* glycosylates the C-3 hydroxyl with mycarose, the C-5 hydroxyl with desosamine, and the C-6 hydroxyl with megosamine,
15 and hydroxylates the C-6 position. *S. antibioticus* produces oleandomycin and contains enzymes that hydroxylate the C-6 and C-12 positions, glycosylate the C-3 hydroxyl with oleandrose and the C-5 hydroxyl with desosamine, and form an epoxide at C-8-C-8a. *S. fradiae* contains enzymes that glycosylate the C-5 hydroxyl with mycaminose and then the 4'-hydroxyl of mycaminose with
20 mycarose, forming a disaccharide. *S. thermotolerans* contains the same activities as *S. fradiae*, as well as acylation activities. Thus, the present invention provides the compounds produced by hydroxylation and glycosylation of the macrolide aglycones of the invention by action of the enzymes endogenous to *M. megalomicea*, *S. antibioticus*, *S. fradiae*, and *S.*
25 *thermotolerans*.

The present invention also provides methods and genetic constructs for producing the glycosylated and/or hydroxylated compounds of the invention directly in the host cell of interest. Thus, the genes that encode polyketide modification enzymes can be included in the host cells of the
30 invention. Lack of adequate resistance to a polyketide can be overcome by providing the host cell with an MLS resistance gene (*ermE* and *mgt/lrm*, for

example), which confer resistance to several 14-membered macrolides (see Cundliffe, 1989, *Annu. Rev. Microbiol.* 43:207-33; Jenkins and Cundliffe, 1991, *Gene* 108:55-62; and Cundliffe, 1992, *Gene*, 115:75-84, each of which is incorporated herein by reference).

5 The recombinant host cells of the invention can be used to produce polyketides (both macrolide aglycones and their modified derivatives) that are naturally occurring or produced by recombinant DNA technology. In one important embodiment, the recombinant host cells of the invention are used to produce hybrid PKS enzymes. For purposes of the invention, a hybrid PKS
10 is a recombinant PKS that comprises all or part of one or more extender modules, loading module, and/or thioesterase/cyclase domain of a first PKS and all or part of one or more extender modules, loading module, and/or thioesterase/cyclase domain of a second PKS.

Those of skill in the art will recognize that all or part of either the first
15 or second PKS in a hybrid PKS of the invention need not be isolated from a naturally occurring source. For example, only a small portion of an AT domain determines its specificity. See PCT patent application No. WO US99/15047, and Lau *et al.*, *infra*, incorporated herein by reference. The state of the art in DNA synthesis allows the artisan to construct *de novo* DNA
20 compounds of size sufficient to construct a useful portion of a PKS module or domain. Thus, the desired derivative coding sequences can be synthesized using standard solid phase synthesis methods such as those described by Jaye *et al.*, 1984, *J. Biol. Chem.* 259: 6331, and instruments for automated synthesis are available commercially from, for example, Applied Biosystems, Inc. For
25 purposes of the invention, such synthetic DNA compounds are deemed to be a portion of a PKS.

A hybrid PKS for purposes of the present invention can result not only:

(i) from fusions of heterologous domain (where heterologous means the domains in a module are derived from at least two different naturally
30 occurring modules) coding sequences to produce a hybrid module coding sequence contained in a PKS gene whose product is incorporated into a PKS,

but also:

- (ii) from fusions of heterologous module (where heterologous module means two modules are adjacent to one another that are not adjacent to one another in naturally occurring PKS enzymes) coding sequences to produce a hybrid coding sequence contained in a PKS gene whose product is incorporated into a PKS,
- (iii) from expression of one or more PKS genes from a first PKS gene cluster with one or more PKS genes from a second PKS gene cluster, and
- (iv) from combinations of the foregoing.

10 Various hybrid PKSs of the invention illustrating these various alternatives are described herein.

Recombinant methods for manipulating modular PKS genes to make hybrid PKS enzymes are described in U.S. Patent Nos. 5,672,491; 5,843,718; 5,830,750; and 5,712,146; and in PCT publication Nos. 98/49315 and 97/02358, each of which is incorporated herein by reference. A number of genetic engineering strategies have been used with DEBS to demonstrate that the structures of polyketides can be manipulated to produce novel natural products, primarily analogs of the erythromycins (see the patent publications referenced *supra* and Hutchinson, 1998, *Curr Opin Microbiol.* 1:319-329, and Baltz, 1998, *Trends Microbiol.* 6:76-83, incorporated herein by reference).

These techniques include: (i) deletion or insertion of modules to control chain length, (ii) inactivation of reduction/dehydration domains to bypass beta-carbon processing steps, (iii) substitution of AT domains to alter starter and extender units, (iv) addition of reduction/dehydration domains to introduce catalytic activities, and (v) substitution of ketoreductase KR domains to control hydroxyl stereochemistry. In addition, engineered blocked mutants of DEBS have been used for precursor directed biosynthesis of analogs that incorporate synthetically derived starter units. For example, more than 100 novel polyketides were produced by engineering single and combinatorial changes in multiple modules of DEBS. Hybrid PKS enzymes based on DEBS with up to three catalytic domain substitutions were

constructed by cassette mutagenesis, in which various DEBS domains were replaced with domains from the rapamycin PKS (see Schweke *et al.*, 1995, *Proc. Nat. Acad. Sci. USA* 92, 7839-7843, incorporated herein by reference) or one more of the DEBS KR domains was deleted. Functional single domain
5 replacements or deletions were combined to generate DEBS enzymes with double and triple catalytic domain substitutions (see McDaniel *et al.*, 1999, *Proc. Nat. Acad. Sci. USA* 96, 1846-1851, incorporated herein by reference).

Methods for generating libraries of polyketides have been greatly improved by cloning PKS genes as a set of three or more mutually selectable
10 plasmids, each carrying a different wild-type or mutant PKS gene, then introducing all possible combinations of the plasmids with wild-type, mutant, and hybrid PKS coding sequences into the same host (see U.S. patent application Serial No. 60/129,731, filed 16 Apr. 1999, and PCT Pub. No. 98/27203, each of which is incorporated herein by reference). This method
15 can also incorporate the use of a KS1° mutant, which by mutational biosynthesis can produce polyketides made from diketide starter units (see Jacobsen *et al.*, 1997, *Science* 277, 367-369, incorporated herein by reference), as well as the use of a truncated gene that leads to 12-membered macrolides or an elongated gene that leads to 16-membered ketolides. Moreover, by
20 utilizing in addition one or more vectors that encode glycosyl biosynthesis and transfer genes, such as those of the present invention for megosamine, desosamine, oleandrose, cladinoses, and/or mycarose (in any combination), a large collection of glycosylated polyketides can be prepared.

The following table lists references describing illustrative PKS genes
25 and corresponding enzymes that can be utilized in the construction of the recombinant hybrid PKSs and the corresponding DNA compounds that encode them. Also presented are various references describing tailoring enzymes and corresponding genes that can be employed in accordance with the methods of the invention.

30 **Avermectin**

U.S. Pat. No. 5,252,474 to Merck.

- MacNeil *et al.*, 1993, Industrial Microorganisms: Basic and Applied Molecular Genetics, Baltz, Hegeman, & Skatrud, eds. (ASM), pp. 245-256, A Comparison of the Genes Encoding the Polyketide Synthases for Avermectin, Erythromycin, and Nemadectin.
- 5 MacNeil *et al.*, 1992, *Gene* 115: 119-125, Complex Organization of the *Streptomyces avermitilis* genes encoding the avermectin polyketide synthase.
- Candicidin (FR008)**
Hu *et al.*, 1994, *Mol. Microbiol.* 14: 163-172.
- Epothilone**
10 PCT Pat. Pub. No. WO 00/031247 to Kosan.
- Erythromycin**
PCT Pub. No. 93/13663 to Abbott.
US Pat. No. 5,824,513 to Abbott.
Donadio *et al.*, 1991, *Science* 252:675-9.
- 15 Cortes *et al.*, 8 Nov. 1990, *Nature* 348:176-8, An unusually large multifunctional polypeptide in the erythromycin producing polyketide synthase of *Saccharopolyspora erythraea*.
- Glycosylation Enzymes
PCT Pat. App. Pub. No. 97/23630 to Abbott.
- 20 **FK-506**
Motamedi *et al.*, 1998, The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK506, *Eur. J. biochem.* 256: 528-534.
- 25 Motamedi *et al.*, 1997, Structural organization of a multifunctional polyketide synthase involved in the biosynthesis of the macrolide immunosuppressant FK506, *Eur. J. Biochem.* 244: 74-80.
- Methyltransferase
US 5,264,355, issued 23 Nov. 1993, Methylating enzyme from *Streptomyces* MA6858. 31-O-desmethyl-FK506 methyltransferase.
- 30 Motamedi *et al.*, 1996, Characterization of methyltransferase and hydroxylase genes involved in the biosynthesis of the immunosuppressants FK506 and FK520, *J. Bacteriol.* 178: 5243-5248.
- FK-520**
PCT Pat. Pub. No. WO 00/020601 to Kosan.
- 35 See also Nielsen *et al.*, 1991, *Biochem.* 30:5789-96 (enzymology of pipercolate incorporation).
- Lovastatin**
U.S. Pat. No. 5,744,350 to Merck.
- Narbomycin (and Picromycin)**
40 PCT Pat. Pub. No. WO 99/61599 to Kosan.
- Nemadectin**
MacNeil *et al.*, 1993, *supra*.
- Niddamycin**
Kakavas *et al.*, 1997, Identification and characterization of the
45 niddamycin polyketide synthase genes from *Streptomyces caelestis*, *J. Bacteriol.* 179: 7515-7522.

Oleandomycin

Swan *et al.*, 1994, Characterisation of a *Streptomyces antibioticus* gene encoding a type I polyketide synthase which has an unusual coding sequence, *Mol. Gen. Genet.* 242: 358-362.

5 PCT Pat. Pub. No. WO 00/026349 to Kosan.

Olano *et al.*, 1998, Analysis of a *Streptomyces antibioticus* chromosomal region involved in oleandomycin biosynthesis, which encodes two glycosyltransferases responsible for glycosylation of the macrolactone ring, *Mol. Gen. Genet.* 259(3): 299-308.

10 **Platenolide**

EP Pat. App. Pub. No. 791,656 to Lilly.

Rapamycin

Schwecke *et al.*, Aug. 1995, The biosynthetic gene cluster for the polyketide rapamycin, *Proc. Natl. Acad. Sci. USA* 92:7839-7843.

15 Aparicio *et al.*, 1996, Organization of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of the enzymatic domains in the modular polyketide synthase, *Gene* 169: 9-16.

Rifamycin

20 August *et al.*, 13 Feb. 1998, Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of *Amycolatopsis mediterranei* S669, *Chemistry & Biology*, 5(2): 69-79.

Soraphen

U.S. Pat. No. 5,716,849 to Novartis.

25 Schupp *et al.*, 1995, *J. Bacteriology* 177: 3673-3679. A *Sorangium cellulosum* (Myxobacterium) Gene Cluster for the Biosynthesis of the Macrolide Antibiotic Soraphen A: Cloning, Characterization, and Homology to Polyketide Synthase Genes from Actinomycetes.

Spiramycin

U.S. Pat. No. 5,098,837 to Lilly.

30 Activator Gene

U.S. Pat. No. 5,514,544 to Lilly.

Tylosin

EP Pub. No. 791,655 to Lilly.

35 Kuhstoss *et al.*, 1996, *Gene* 183:231-6., Production of a novel polyketide through the construction of a hybrid polyketide synthase.

U.S. Pat. No. 5,876,991 to Lilly.

Tailoring enzymes

Merson-Davies and Cundliffe, 1994, *Mol. Microbiol.* 13: 349-355.

40 Analysis of five tylosin biosynthetic genes from the *tylBA* region of the *Streptomyces fradiae* genome.

As the above Table illustrates, there are a wide variety of PKS genes that serve as readily available sources of DNA and sequence information for use in constructing the hybrid PKS-encoding DNA compounds of the invention.

In constructing hybrid PKSs, certain general methods may be helpful. For example, it is often beneficial to retain the framework of the module to be altered to make the hybrid PKS. Thus, if one desires to add DH and ER functionalities to a module, it is often preferred to replace the KR domain of the original module with a KR, DH, and ER domain-containing segment from another module, instead of merely inserting DH and ER domains. One can alter the stereochemical specificity of a module by replacement of the KS domain with a KS domain from a module that specifies a different stereochemistry. See Lau *et al.*, 1999, "Dissecting the role of acyltransferase domains of modular polyketide synthases in the choice and stereochemical fate of extender units" *Biochemistry* 38(5):1643-1651, incorporated herein by reference. One can alter the specificity of an AT domain by changing only a small segment of the domain. See Lau *et al.*, *supra*. One can also take advantage of known linker regions in PKS proteins to link modules from two different PKSs to create a hybrid PKS. See Gokhale *et al.*, 16 Apr. 1999, "Dissecting and Exploiting Intermodular Communication in Polyketide Synthases", *Science* 284: 482-485, incorporated herein by reference.

The hybrid PKS-encoding DNA compounds can be and often are hybrids of more than two PKS genes. Even where only two genes are used, there are often two or more modules in the hybrid gene in which all or part of the module is derived from a second (or third) PKS gene.

The invention also provides libraries of PKS genes, PKS proteins, and ultimately, of polyketides, that are constructed by generating modifications in a PKS so that the protein complexes produced have altered activities in one or more respects and thus produce polyketides other than the natural product of the PKS. Novel polyketides may thus be prepared, or polyketides in general prepared more readily, using this method. By providing a large number of different genes or gene clusters derived from a naturally occurring PKS gene cluster, each of which has been modified in a different way from the native cluster, an effectively combinatorial library of polyketides can be produced as a result of the multiple variations in these activities. As will be

further described below, the metes and bounds of this embodiment of the invention can be described on the polyketide, protein, and the encoding nucleotide sequence levels.

There are at least five degrees of freedom for constructing a hybrid
5 PKS in terms of the polyketide that will be produced. First, the polyketide chain length is determined by the number of extender modules in the PKS, and the present invention includes hybrid PKSs that contain 6, as well as fewer or more than 6, extender modules. Second, the nature of the carbon skeleton of the PKS is determined by the specificities of the acyl transferases
10 that determine the nature of the extender units at each position, e.g., malonyl, methylmalonyl, ethylmalonyl, or other substituted malonyl. Third, the loading module specificity also has an effect on the resulting carbon skeleton of the polyketide. The loading module may use a different starter unit, such as acetyl, butyryl, and the like. As noted above, another method for varying
15 loading module specificity involves inactivating the KS activity in extender module 1 (KS1) and providing alternative substrates, called diketides, that are chemically synthesized analogs of extender module 1 diketide products, for extender module 2. This approach was illustrated in PCT publication Nos. 97/02358 and 99/03986, incorporated herein by reference, wherein the KS1
20 activity was inactivated through mutation. Fourth, the oxidation state at various positions of the polyketide will be determined by the dehydratase and reductase portions of the modules. This will determine the presence and location of ketone and alcohol moieties and C-C double bonds or C-C single bonds in the polyketide. Finally, the stereochemistry of the resulting
25 polyketide is a function of three aspects of the synthase. The first aspect is related to the AT/KS specificity associated with substituted malonyls as extender units, which affects stereochemistry only when the reductive cycle is missing or when it contains only a ketoreductase, as the dehydratase would abolish chirality. Second, the specificity of the ketoreductase may determine
30 the chirality of any beta-OH. Finally, the enoylreductase specificity for

substituted malonyls as extender units may influence the stereochemistry when there is a complete KR/DH/ER available.

Thus, the modular PKS systems generally permit a wide range of polyketides to be synthesized. As compared to the aromatic PKS systems, the modular PKS systems accept a wider range of starter units, including aliphatic monomers (acetyl, propionyl, butyryl, isovaleryl, etc.), aromatics (aminohydroxybenzoyl), alicyclics (cyclohexanoyl), and heterocyclics (thiazolyl). Certain modular PKSs have relaxed specificity for their starter units (Kao *et al.*, 1994, *Science, supra*). Modular PKSs also exhibit considerable variety with regard to the choice of extender units in each condensation cycle. The degree of beta-ketoreduction following a condensation reaction can be altered by genetic manipulation (Donadio *et al.*, 1991, *Science, supra*; Donadio *et al.*, 1993, *Proc. Natl. Acad. Sci. USA* 90: 7119-7123). Likewise, the size of the polyketide product can be varied by designing mutants with the appropriate number of modules (Kao *et al.*, 1994, *J. Am. Chem. Soc.* 116:11612-11613). Lastly, modular PKS enzymes are particularly well known for generating an impressive range of asymmetric centers in their products in a highly controlled manner. The polyketides, antibiotics, and other compounds produced by the methods of the invention are typically single stereoisomeric forms. Although the compounds of the invention can occur as mixtures of stereoisomers, it may be beneficial in some instances to generate individual stereoisomers. Thus, the combinatorial potential within modular PKS pathways based on any naturally occurring modular PKS scaffold is virtually unlimited.

While hybrid PKSs are most often produced by "mixing and matching" portions of PKS coding sequences, mutations in DNA encoding a PKS can also be used to introduce, alter, or delete an activity in the encoded polypeptide. Mutations can be made to the native sequences using conventional techniques. The substrates for mutation can be an entire cluster of genes or only one or two of them; the substrate for mutation may also be portions of one or more of these genes. Techniques for mutation include

- preparing synthetic oligonucleotides including the mutations and inserting the mutated sequence into the gene encoding a PKS subunit using restriction endonuclease digestion. See, e.g., Kunkel, 1985, *Proc. Natl. Acad. Sci. USA* 82: 448; Geisselsoder *et al.*, 1987, *BioTechniques* 5:786. Alternatively, the mutations
- 5 can be effected using a mismatched primer (generally 10-20 nucleotides in length) that hybridizes to the native nucleotide sequence, at a temperature below the melting temperature of the mismatched duplex. The primer can be made specific by keeping primer length and base composition within relatively narrow limits and by keeping the mutant base centrally located.
- 10 See Zoller and Smith, 1983, *Methods Enzymol.* 100:468. Primer extension is effected using DNA polymerase, the product cloned, and clones containing the mutated DNA, derived by segregation of the primer extended strand, selected. Identification can be accomplished using the mutant primer as a hybridization probe. The technique is also applicable for generating multiple
- 15 point mutations. See, e.g., Dalbie-McFarland *et al.*, 1982, *Proc. Natl. Acad. Sci. USA* 79: 6409. PCR mutagenesis can also be used to effect the desired mutations.

Random mutagenesis of selected portions of the nucleotide sequences encoding enzymatic activities can also be accomplished by several different

20 techniques known in the art, e.g., by inserting an oligonucleotide linker randomly into a plasmid, by irradiation with X-rays or ultraviolet light, by incorporating incorrect nucleotides during *in vitro* DNA synthesis, by error-prone PCR mutagenesis, by preparing synthetic mutants, or by damaging plasmid DNA *in vitro* with chemicals. Chemical mutagens include, for

25 example, sodium bisulfite, nitrous acid, nitrosoguanidine, hydroxylamine, agents which damage or remove bases thereby preventing normal base-pairing such as hydrazine or formic acid, analogues of nucleotide precursors such as 5-bromouracil, 2-aminopurine, or acridine intercalating agents such as proflavine, acriflavine, quinacrine, and the like. Generally, plasmid DNA or

30 DNA fragments are treated with chemical mutagens, transformed into *E. coli* and propagated as a pool or library of mutant plasmids.

In constructing a hybrid PKS of the invention, regions encoding enzymatic activity, i.e., regions encoding corresponding activities from different PKS synthases or from different locations in the same PKS, can be recovered, for example, using PCR techniques with appropriate primers. By
5 "corresponding" activity encoding regions is meant those regions encoding the same general type of activity. For example, a KR activity encoded at one location of a gene cluster "corresponds" to a KR encoding activity in another location in the gene cluster or in a different gene cluster. Similarly, a complete reductase cycle could be considered corresponding. For example,
10 KR/DH/ER can correspond to a KR alone.

If replacement of a particular target region in a host PKS is to be made, this replacement can be conducted *in vitro* using suitable restriction enzymes. The replacement can also be effected *in vivo* using recombinant techniques involving homologous sequences framing the replacement gene in a donor
15 plasmid and a receptor region in a recipient plasmid. Such systems, advantageously involving plasmids of differing temperature sensitivities are described, for example, in PCT publication No. WO 96/40968, incorporated herein by reference. The vectors used to perform the various operations to replace the enzymatic activity in the host PKS genes or to support mutations
20 in these regions of the host PKS genes can be chosen to contain control sequences operably linked to the resulting coding sequences in a manner such that expression of the coding sequences can be effected in an appropriate host.

However, simple cloning vectors may be used as well. If the cloning vectors employed to obtain PKS genes encoding derived PKS lack control
25 sequences for expression operably linked to the encoding nucleotide sequences, the nucleotide sequences are inserted into appropriate expression vectors. This need not be done individually, but a pool of isolated encoding nucleotide sequences can be inserted into expression vectors, the resulting vectors transformed or transfected into host cells, and the resulting cells
30 plated out into individual colonies. The invention provides a variety of recombinant DNA compounds in which the various coding sequences for the

domains and modules of the PKS are flanked by non-naturally occurring restriction enzyme recognition sites.

The various PKS nucleotide sequences can be cloned into one or more recombinant vectors as individual cassettes, with separate control elements, or under the control of, e.g., a single promoter. The PKS subunit encoding regions can include flanking restriction sites to allow for the easy deletion and insertion of other PKS subunit encoding sequences so that hybrid PKSs can be generated. The design of such unique restriction sites is known to those of skill in the art and can be accomplished using the techniques described above, such as site-directed mutagenesis and PCR.

The expression vectors containing nucleotide sequences encoding a variety of PKS enzymes for the production of different polyketides are then transformed into the appropriate host cells to construct the library. In one straightforward approach, a mixture of such vectors is transformed into the selected host cells and the resulting cells plated into individual colonies and selected to identify successful transformants. Each individual colony has the ability to produce a particular PKS synthase and ultimately a particular polyketide. Typically, there will be duplications in some, most, or all of the colonies; the subset of the transformed colonies that contains a different PKS in each member colony can be considered the library. Alternatively, the expression vectors can be used individually to transform hosts, which transformed hosts are then assembled into a library. A variety of strategies are available to obtain a multiplicity of colonies each containing a PKS gene cluster derived from the naturally occurring host gene cluster so that each colony in the library produces a different PKS and ultimately a different polyketide. The number of different polyketides that are produced by the library is typically at least four, more typically at least ten, and preferably at least 20, and more preferably at least 50, reflecting similar numbers of different altered PKS gene clusters and PKS gene products. The number of members in the library is arbitrarily chosen; however, the degrees of freedom outlined above with respect to the variation of starter, extender units,

stereochemistry, oxidation state, and chain length enables the production of quite large libraries.

Methods for introducing the recombinant vectors of the invention into suitable hosts are known to those of skill in the art and typically include the use of CaCl_2 or agents such as other divalent cations, lipofection, DMSO, protoplast transformation, infection, transfection, and electroporation. The polyketide producing colonies can be identified and isolated using known techniques and the produced polyketides further characterized. The polyketides produced by these colonies can be used collectively in a panel to represent a library or may be assessed individually for activity.

The libraries of the invention can thus be considered at four levels: (1) a multiplicity of colonies each with a different PKS encoding sequence; (2) the proteins produced from the coding sequences; (3) the polyketides produced from the proteins assembled into a function PKS; and (4) antibiotics or compounds with other desired activities derived from the polyketides.

Colonies in the library are induced to produce the relevant synthases and thus to produce the relevant polyketides to obtain a library of polyketides. The polyketides secreted into the media can be screened for binding to desired targets, such as receptors, signaling proteins, and the like. The supernatants *per se* can be used for screening, or partial or complete purification of the polyketides can first be effected. Typically, such screening methods involve detecting the binding of each member of the library to receptor or other target ligand. Binding can be detected either directly or through a competition assay. Means to screen such libraries for binding are well known in the art. Alternatively, individual polyketide members of the library can be tested against a desired target. In this event, screens wherein the biological response of the target is measured can more readily be included. Antibiotic activity can be verified using typical screening assays such as those set forth in Lehrer *et al.*, 1991, *J. Immunol. Meth.* 137:167-173, incorporated herein by reference, and in the Examples below.

The invention provides methods for the preparation of a large number of polyketides. These polyketides are useful intermediates in formation of compounds with antibiotic or other activity through hydroxylation, epoxidation, and glycosylation reactions as described above. In general, the polyketide products of the PKS must be further modified, typically by hydroxylation and glycosylation, to exhibit antibiotic activity. Hydroxylation results in the novel polyketides of the invention that contain hydroxyl groups at C-6, which can be accomplished using the hydroxylase encoded by the *eryF* gene, and/or C-12, which can be accomplished using the hydroxylase encoded by the *picK* or *eryK* gene. Also, the *oleP* gene is available in recombinant form, which can be used to express the *oleP* gene product in any host cell. A host cell, such as a *Streptomyces* host cell or a *Saccharopolyspora erythraea* host cell, modified to express the *oleP* gene thus can be used to produce polyketides comprising the C-8-C-8a epoxide present in oleandomycin. Thus the invention provides such modified polyketides. The presence of hydroxyl groups at these positions can enhance the antibiotic activity of the resulting compound relative to its unhydroxylated counterpart.

Methods for glycosylating the polyketides are generally known in the art; the glycosylation may be effected intracellularly by providing the appropriate glycosylation enzymes or may be effected *in vitro* using chemical synthetic means as described herein and in PCT publication No. WO 98/49315, incorporated herein by reference. Preferably, glycosylation with desosamine, mycarose, and/or megosamine is effected in accordance with the methods of the invention in recombinant host cells provided by the invention. In general, the approaches to effecting glycosylation mirror those described above with respect to hydroxylation. The purified enzymes, isolated from native sources or recombinantly produced may be used *in vitro*. Alternatively and as noted, glycosylation may be effected intracellularly using endogenous or recombinantly produced intracellular glycosylases. In addition, synthetic chemical methods may be employed.

The antibiotic modular polyketides may contain any of a number of different sugars, although D-desosamine, or a close analog thereof, is most common. Erythromycin, picromycin, megalomicin, narbomycin, and methymycin contain desosamine. Erythromycin also contains L-cladinose (3-O-methyl mycarose). Tylosin contains mycaminoses (4-hydroxy desosamine), mycarose and 6-deoxy-D-allose. 2-acetyl-1-bromodesosamine has been used as a donor to glycosylate polyketides by Masamune *et al.*, 1975, *J. Am. Chem. Soc.* 97: 3512-3513. Other, apparently more stable donors include glycosyl fluorides, thioglycosides, and trichloroacetimidates; see Woodward *et al.*, 1981, *J. Am. Chem. Soc.* 103: 3215; Martin *et al.*, 1997, *J. Am. Chem. Soc.* 119: 3193; Toshima *et al.*, 1995, *J. Am. Chem. Soc.* 117: 3717; Matsumoto *et al.*, 1988, *Tetrahedron Lett.* 29: 3575. Glycosylation can also be effected using the polyketide aglycones as starting materials and using *Saccharopolyspora erythraea* or *Streptomyces venezuelae* or other host cell to make the conversion, preferably using mutants unable to synthesize macrolides, as discussed above.

Thus, a wide variety of polyketides can be produced by the hybrid PKS enzymes of the invention. These polyketides are useful as antibiotics and as intermediates in the synthesis of other useful compounds. In one important aspect, the invention provides methods for making antibiotic compounds related in structure to erythromycin, a potent antibiotic compound. The invention also provides novel ketolide compounds, polyketide compounds with potent antibiotic activity of significant interest due to activity against antibiotic resistant strains of bacteria. See Griesgraber *et al.*, 1996, *J. Antibiot.* 49: 465-477, incorporated herein by reference. Most if not all of the ketolides prepared to date are synthesized using erythromycin A, a derivative of 6-dEB, as an intermediate. See Griesgraber *et al.*, *supra*; Agouridas *et al.*, 1998, *J. Med. Chem.* 41: 4080-4100, U.S. Patent Nos. 5,770,579; 5,760,233; 5,750,510; 5,747,467; 5,747,466; 5,656,607; 5,635,485; 5,614,614; 5,556,118; 5,543,400; 5,527,780; 5,444,051; 5,439,890; 5,439,889; and PCT publication Nos. WO 98/09978 and 98/28316, each of which is incorporated herein by reference.

As noted above, the hybrid PKS genes of the invention can be expressed in a host cell that contains the desosamine, megosamine, and/or mycarose biosynthetic genes and corresponding transferase genes as well as the required hydroxylase gene(s), which may be either *picK*, *megK*, or *eryK* (for the C-12 position) and/or *megF* or *eryF* (for the C-6 position). The resulting compounds have antibiotic activity but can be further modified, as described in the patent publications referenced above, to yield a desired compound with improved or otherwise desired properties. Alternatively, the aglycone compounds can be produced in the recombinant host cell, and the desired glycosylation and hydroxylation steps carried out *in vitro* or *in vivo*, in the latter case by supplying the converting cell with the aglycone, as described above.

As described above, there are a wide variety of diverse organisms that can modify compounds such as those described herein to provide compounds with or that can be readily modified to have useful activities. For example, *Saccharopolyspora erythraea* can convert 6-dEB to a variety of useful compounds. The compounds provided by the present invention can be provided to cultures of *Saccharopolyspora erythraea* and converted to the corresponding derivatives of erythromycins A, B, C, and D in accordance with the procedure provided in the Examples, below. To ensure that only the desired compound is produced, one can use an *S. erythraea eryA* mutant that is unable to produce 6-dEB but can still carry out the desired conversions (Weber *et al.*, 1985, *J. Bacteriol.* 164(1): 425-433). Also, one can employ other mutant strains, such as *eryB*, *eryC*, *eryG*, and/or *eryK* mutants, or mutant strains having mutations in multiple genes, to accumulate a preferred compound. The conversion can also be carried out in large fermentors for commercial production. Each of the erythromycins A, B, C, and D has antibiotic activity, although erythromycin A has the highest antibiotic activity. Moreover, each of these compounds can form, under treatment with mild acid, a C-6 to C-9 hemiketal with motilide activity. For formation of hemiketals with motilide activity, erythromycins B, C, and D, are preferred, as

the presence of a C-12 hydroxyl allows the formation of an inactive compound that has a hemiketal formed between C-9 and C-12.

Thus, the present invention provides the compounds produced by hydroxylation and glycosylation of the compounds of the invention by action of the enzymes endogenous to *Saccharopolyspora erythraea* and mutant strains of *S. erythraea*. Such compounds are useful as antibiotics or as motilides directly or after chemical modification. For use as antibiotics, the compounds of the invention can be used directly without further chemical modification. Erythromycins A, B, C, and D all have antibiotic activity, and the corresponding compounds of the invention that result from the compounds being modified by *Saccharopolyspora erythraea* also have antibiotic activity. These compounds can be chemically modified, however, to provide other compounds of the invention with potent antibiotic activity. For example, alkylation of erythromycin at the C-6 hydroxyl can be used to produce potent antibiotics (clarithromycin is C-6-O-methyl), and other useful modifications are described in, for example, Griesgraber *et al.*, 1996, *J. Antibiot.* 49: 465-477, Agouridas *et al.*, 1998, *J. Med. Chem.* 41: 4080-4100, U.S. Patent Nos. 5,770,579; 5,760,233; 5,750,510; 5,747,467; 5,747,466; 5,656,607; 5,635,485; 5,614,614; 5,556,118; 5,543,400; 5,527,780; 5,444,051; 5,439,890; and 5,439,889; and PCT publication Nos. WO 98/09978 and 98/28316, each of which is incorporated herein by reference.

For use as motilides, the compounds of the invention can be used directly without further chemical modification. Erythromycin and certain erythromycin analogs are potent agonists of the motilin receptor that can be used clinically as prokinetic agents to induce phase III of migrating motor complexes, to increase esophageal peristalsis and LES pressure in patients with GERD, to accelerate gastric emptying in patients with gastric paresis, and to stimulate gall bladder contractions in patients after gallstone removal and in diabetics with autonomic neuropathy. See Peeters, 1999, Motilide Web Site, <http://www.med.kuleuven.ac.be/med/gih/motilid.htm>, and Omura *et al.*, 1987, Macrolides with gastrointestinal motor stimulating activity, *J. Med.*

Chem. 30: 1941-3). The corresponding compounds of the invention that result from the compounds of the invention being modified by *Saccharopolyspora erythraea* also have motilide activity, particularly after conversion, which can also occur *in vivo*, to the C-6 to C-9 hemiketal by treatment with mild acid.

- 5 Compounds lacking the C-12 hydroxyl are especially preferred for use as motilin agonists. These compounds can also be further chemically modified, however, to provide other compounds of the invention with potent motilide activity.

- Moreover, and also as noted above, there are other useful organisms
10 that can be employed to hydroxylate and/or glycosylate the compounds of the invention. As described above, the organisms can be mutants unable to produce the polyketide normally produced in that organism, the fermentation can be carried out on plates or in large fermentors, and the compounds produced can be chemically altered after fermentation. In addition to
15 *Saccharopolyspora erythraea*, *Streptomyces venezuelae*, *S. narbonensis*, *S. antibioticus*, *Micromonospora megalomicea*, *S. fradiae*, and *S. thermotolerans* can also be used. In addition to antibiotic activity, compounds of the invention produced by treatment with *M. megalomicea* enzymes can have antiparasitic activity as well. Thus, the present invention provides the compounds
20 produced by hydroxylation and glycosylation by action of the enzymes endogenous to *S. erythraea*, *S. venezuelae*, *S. narbonensis*, *S. antibioticus*, *M. megalomicea*, *S. fradiae*, and *S. thermotolerans*.

- The compounds of the invention can be isolated from the fermentation
broths of these cultured cells and purified by standard procedures. The
25 compounds can be readily formulated to provide the pharmaceutical compositions of the invention. The pharmaceutical compositions of the invention can be used in the form of a pharmaceutical preparation, for example, in solid, semisolid, or liquid form. This preparation will contain one or more of the compounds of the invention as an active ingredient in
30 admixture with an organic or inorganic carrier or excipient suitable for external, enteral, or parenteral application. The active ingredient may be

compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use.

The carriers which can be used include water, glucose, lactose, gum
5 acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, and other carriers suitable for use in manufacturing preparations, in solid, semi-solid, or liquified form. In addition, auxiliary stabilizing, thickening, and coloring agents and perfumes may be used. For example, the compounds of the invention may be utilized
10 with hydroxypropyl methylcellulose essentially as described in U.S. Patent No. 4,916,138, incorporated herein by reference, or with a surfactant essentially as described in EPO patent publication No. 428,169, incorporated herein by reference.

Oral dosage forms may be prepared essentially as described by Hondo
15 *et al.*, 1987, *Transplantation Proceedings XIX*, Supp. 6: 17-22, incorporated herein by reference. Dosage forms for external application may be prepared essentially as described in EPO patent publication No. 423,714, incorporated herein by reference. The active compound is included in the pharmaceutical composition in an amount sufficient to produce the desired effect upon the
20 disease process or condition.

For the treatment of conditions and diseases caused by infection, a compound of the invention may be administered orally, topically, parenterally, by inhalation spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers,
25 adjuvant, and vehicles. The term parenteral, as used herein, includes subcutaneous injections, and intravenous, intramuscular, and intrasternal injection or infusion techniques.

Dosage levels of the compounds of the invention are of the order from about 0.01 mg to about 50 mg per kilogram of body weight per day,
30 preferably from about 0.1 mg to about 10 mg per kilogram of body weight per day. The dosage levels are useful in the treatment of the above-indicated

conditions (from about 0.7 mg to about 3.5 mg per patient per day, assuming a 70 kg patient). In addition, the compounds of the invention may be administered on an intermittent basis, i.e., at semi-weekly, weekly, semi-monthly, or monthly intervals.

5 The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. For example, a formulation intended for oral administration to humans may contain from 0.5 mg to 5 gm of active agent compounded with an appropriate and convenient
10 amount of carrier material, which may vary from about 5 percent to about 95 percent of the total composition. Dosage unit forms will generally contain from about 0.5 mg to about 500 mg of active ingredient. For external administration, the compounds of the invention may be formulated within the range of, for example, 0.00001% to 60% by weight, preferably from 0.001%
15 to 10% by weight, and most preferably from about 0.005% to 0.8% by weight.

It will be understood, however, that the specific dose level for any particular patient will depend on a variety of factors. These factors include the activity of the specific compound employed; the age, body weight, general health, sex, and diet of the subject; the time and route of administration and
20 the rate of excretion of the drug; whether a drug combination is employed in the treatment; and the severity of the particular disease or condition for which therapy is sought.

A detailed description of the invention having been provided above, the following examples are given for the purpose of illustrating the invention
25 and shall not be construed as being a limitation on the scope of the invention or claims.

Example 1

Production of Methylmalonyl-CoA in *E. Coli*

This example describes, in part A, the cloning and expression of methylmalonyl-CoA mutase, and in part B, the cloning and expression of methylmalonyl-CoA epimerase, in *E. coli*.

A. Cloning and expression of methylmalonyl-CoA mutase

Methylmalonyl-CoA mutase was cloned from *Propionibacterium shermanii* and expressed in *E. coli*. The holoenzyme mm-CoA mutase was obtained by growing cells in the presence of hydroxocobalamin and was shown to be active without addition of vitamin B12. Methylmalonyl-CoA was produced *in vivo*, as seen by CoA analysis using a *panD* strain of BL21 (DE3).

To support modular polyketide production in *E. coli*, the invention provides methods and reagents to produce (S)-methylmalonyl-CoA, which is not naturally present in *E. coli*, by overexpressing mm-CoA mutase and mm-CoA epimerase in *E. coli*. An active, FLAG-tagged version of the mm-CoA mutase from *S. cinnamonensis* was expressed in XL1Blue cells, which were grown in the presence of hydroxocobalamin in a synthetic, vitamin-free media to produce active holoenzyme. The CoA levels in the cells were analyzed by feeding labeled β -alanine; for this purpose it is beneficial to have a *panD* strain, which is a β -alanine auxotroph. The mutase DNA rearranged in the *panD* strain of SJ16, a *recA*⁺ strain, such that the CoA analysis had to be carried out without the *panD*. This resulted in a lower signal to noise ratio, but elevated mm-CoA levels could still be detected. As an alternative to the *S. cinnamonensis* genes, the invention provides a mm-CoA mutase from *P. shermanii* cloned into an *E. coli* expression vector, which is active without addition of vitamin B12, and which elevates mm-CoA levels in *E. coli* in a *panD* strain compatible with the mutase DNA.

Propionibacterium freudenreichii subsp. *shermanii* was obtained as a stab in tomato juice agar from derived from a freeze-dried specimen from NCIMB, Scotland (NCIMB # 9885). *E. coli* strain gg3, a *panD* version of BL21 (DE3)

was used for the CoA analysis. *E. coli* strains gg1 and gg2, recA⁻ versions of the SJ16 *panD* strain, were also used. The vector pKK** is a version of pKK223-3 in which the cloning region is altered to range from *Nde*I to *Eco*R1 and an extra *Nde*I site is deleted. Growth of *P. shermanii* and preparation of
5 genomic DNA was conducted as described in the literature.

Subcloning of methylmalonyl-CoA mutase from *P. shermanii* into *E. coli* was conducted as follows. The gene for mm-CoA mutase consists of two subunits, *mutA* and *mutB*, which were amplified by PCR from *P. shermanii* genomic DNA in a total of four fragments. Naturally occurring restriction
10 sites were used to piece the gene together. Unique restriction sites were introduced at both ends of the gene for cloning purposes, and the start codon for the *mutB* gene was changed from GTG to ATG. As illustrated below, these four fragments were cloned into a BluescriptTM (Stratagene) vector, sequenced, and then pieced together to form the complete mutase gene. The
15 gene was then cloned into expression vectors pET22b and pKK** between the restriction sites *Nde*I and *Hind*III, to form pET-MUT and pKK**-MUT.

The pET-MUT was transformed into competent cells BL21(DE3) and later into cells gg3, which are a *panD* version of BL21(DE3). The pKK**-MUT was transformed into SJ16 *panD* and into XL1Blue. The DNA was tested by
20 screening several colonies with *Nde*I and *Hind*III, to determine if the mutase gene was still present or if it had rearranged.

For SDS-PAGE analysis, cells of strain BL21(DE3) containing pET-MUT (and pET alone, as a control) were grown aerobically at 27°C in MUT media with 100 µg/ml carbenicillin (carb) (MUT media is M9 salts, glucose,
25 thiamine, trace elements and amino acids, as previously described for the expression of methionine synthase (Amaratunga, 1996)). Overnight cultures (250 µl) were used to inoculate 25 mL of MUT media (carb), which were grown at 27°C to an OD₆₀₀ of approximately 0.5. The cultures were then induced with IPTG to 1 mM final concentration. Two cultures were left at
30 27°C for three hours while duplicate cultures were grown at 37°C for two hours. The cells were collected by centrifugation and the pellets were stored

at -80°C prior to analysis. The cells were lysed by sonication and both the soluble and insoluble phases were examined by SDS/PAGE. This procedure was repeated for cells of strain XL1Blue containing pKK**-MUT.

For expression of active mm-CoA mutase (with hydroxocobalamin),
5 cells of strain gg3 containing pET-MUT (and pET alone, as a control) were grown in MUT media (carb) and 5 µM beta-alanine for approximately 20 hours at 27°C. The following operations were performed in a dark room with a red safelight: 125-mL flasks, each containing 25 mL of MUT media with carb and 5 µM β -alanine and wrapped in aluminum foil, were inoculated with 5
10 µM hydroxocobalamin and then with 250 µL from the respective starter cultures. After shaking overnight at 27°C, the cultures were induced with IPTG to 1 mM final concentration and grown for an additional 4:45 hours, at which point they were collected (in Falcon tubes wrapped in aluminum foil) by centrifugation at 4000 rpm for 10 minutes. The pellets were stored in the
15 dark at -80°C prior to assaying.

The mutase assay was performed as follows. All operations were performed in the dark or under a red safelight. The pellet from 25 mL of culture was thawed, washed in buffer C (50 mM potassium phosphate pH 7.4, 5 mM EDTA, 10% glycerol), and resuspended in 0.5 mL of buffer C containing
20 protease inhibitors (1 tablet per 10 mL of buffer). Following sonication on ice, the extract was clarified by centrifugation at 4°C for 10 minutes at maximum speed in an Eppendorf microfuge; the supernatant was assayed. Enzyme assays contained, in a final volume of 100 µL, 0.2 mM (2R,2S)-methylmalonyl-CoA, mutase extract, and buffer C containing protease inhibitors. Reactions
25 for assays with vitamin B12 were as above but contained 0.01 mM vitamin B12, in which case the mutase extract was incubated with the vitamin B12 in a total volume of 75 µL for 5 minutes at 30°C prior to initiation of reaction with methylmalonyl-CoA. After the desired length of incubation at 30°C, the reaction was stopped by the addition of 50 µL of 10% trichloroacetic acid
30 (TCA) and placed on ice for approximately 10 minutes. Cellular debris and precipitated protein were removed by centrifugation for 5 minutes in an

Eppendorf microfuge at 4°C. An aliquot (100 µL) of the supernatant was injected onto the HPLC to quantify conversion of methylmalonyl-CoA to succinyl-CoA. One time point was taken after 20 minutes of incubation at 30°C, and the sample was assayed for conversion of mm-CoA to succinyl-CoA. All operations were performed exclusively under a red safelight until the reaction was stopped by addition of TCA.

The CoA analysis was performed as described in the literature, except that 5 µM of hydroxocobalamin were added at the time of IPTG induction, and the tubes were wrapped in aluminum foil and grown at 27°C instead of 30°C. The CoA peaks, which eluted in approximately one minute each, were collected manually, as well as approximately one minute of sample both before and after each peak. In some tests, fractions were collected every 30 seconds. All samples were counted in the scintillation counter.

The two subunits of the gene encoding methylmalonyl-CoA mutase are translationally coupled – the GTG start codon of the downstream subunit *mutB* overlaps with the ATG codon of *mutA*. The GTG valine start was mutated to an ATG methionine start (which does not alter any other amino acids), because *E. coli* utilizes the methionine start more efficiently. Sequencing the mm-CoA mutase gene revealed a discrepancy between the sequence observed and the published sequence (117-7). A “GC” instead of a “CG” changed two amino acids from Asp,Val to Glu, Leu. The crystal structure of mm-CoA mutase from *P. shermanii* (1996) showed that the two amino acids are indeed Glu, Leu, so the published sequence is in error. The mm-CoA mutase gene was subcloned into two different *E. coli* expression systems: pET, which is under control of the strong T7 promoter, and pKK, which uses the leaky *tac* promoter. First it was necessary to find strains in which the mutase DNA did not rearrange. It was previously observed that a FLAG-tagged version of the mutase from *S. cinnamonensis* rearranged in SJ16 *panD* and in BL21(DE3), which are both *recA*⁺ strains, but not in XL1Blue, which is *recA*⁻. This mutase DNA (*P. shermanii*) also rearranged in the SJ16 cells but not in the BL21(DE3) cells. Thus a *panD* version of BL21(DE3) was

created (gg3) for use with the pET vector. A recA⁻ version of SJ16 was also created (gg1, gg2) for use with the pKK system; however, the mutase DNA rearranged in this strain as well.

5 Different growth conditions were tested to find conditions in which the two subunits of the mutase were expressed in the soluble phase in approximately equal molar ratios. In general, it seemed that the higher temperature of 37°C caused the mutase to appear predominantly in the insoluble form. Growth exclusively at 27°C resulted in soluble protein with an approximately equal subunit ratio.

10 The graph below shows the comparison of *in vivo* acyl-CoA levels in BL21(DE3) *panD* strains with and without mm-CoA mutase. For each CoA, the ratio of the amount in the strain containing the mutase to the amount in the control strain was determined. Interestingly, malonyl-CoA was increased about 25-fold and succinyl-CoA about 3-fold. Acetyl-CoA and CoA were
15 increased just slightly, and propionyl-CoA was not detected in either case.

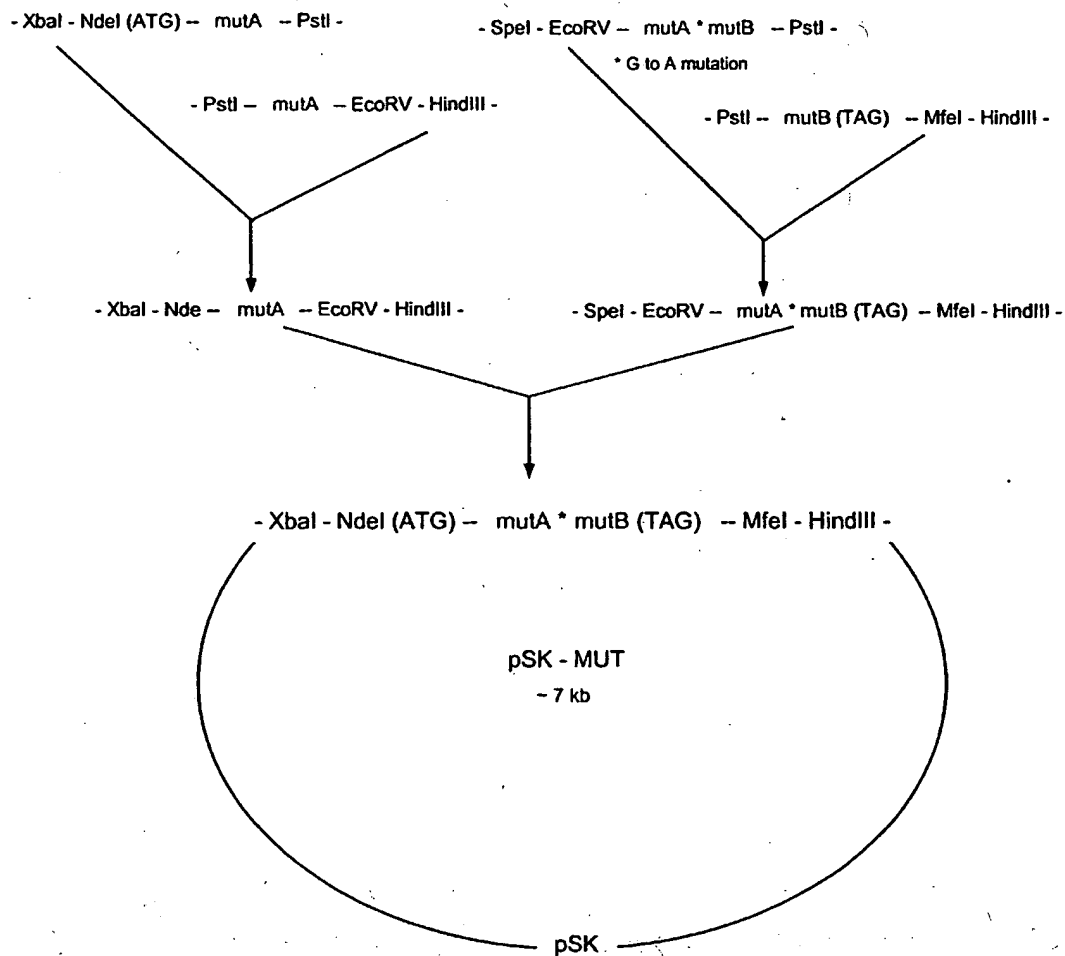
To express active mutase *in vivo*, it was necessary to grow cells in a defined media (MUT media) that allows uptake of the vitamin B12 precursor hydroxocobalamin; this is similar to an established protocol for expression of active methionine synthase, which also requires B12. Cell extracts
20 overexpressing the mutase were shown to convert mm-CoA to succinyl CoA without the addition of vitamin B12. Only one time point (at 20 minutes) was assayed to confirm activity; the specific activity of the mutase must was not determined.

Thus, methylmalonyl-CoA mutase was expressed as the active
25 holoenzyme in *E. coli*, and methylmalonyl-CoA was produced *in vivo*. Because a slow, spontaneous chemical epimerization between (R)- and (S)-mm-CoA does exist (approximately 3% in 15 minutes), it may be helpful to determine the relative amounts of these diastereomers in cells overexpressing the mutase. Enough (S)-mm-CoA may be present to support polyketide
30 production in some cells without addition of an epimerase. To facilitate the

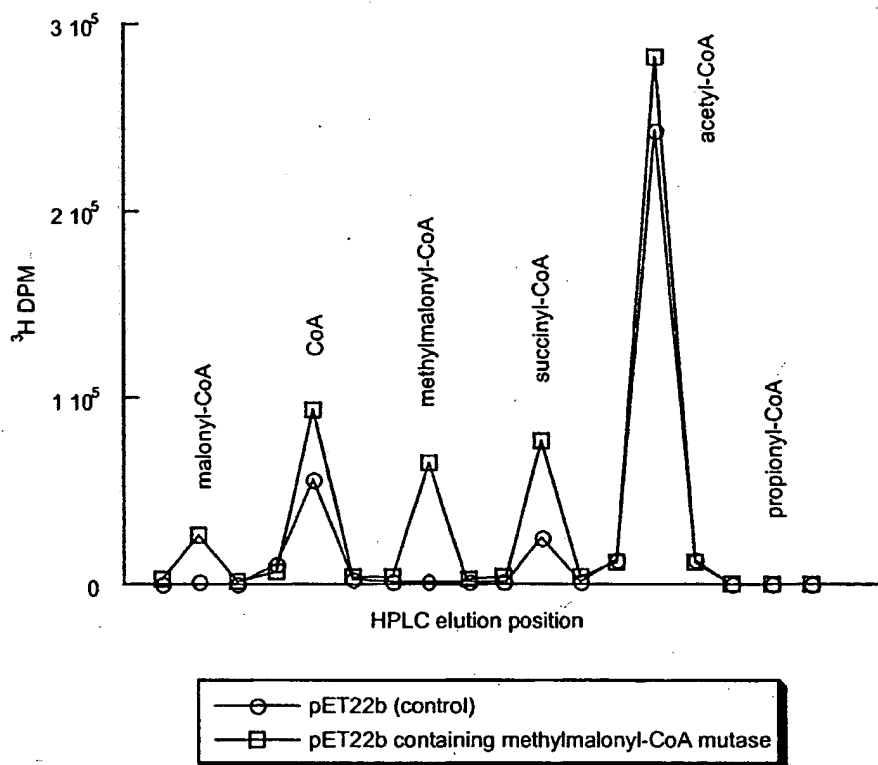
eventual production of polyketides in *E. coli*, the mutase gene can be incorporated into the chromosome of the BL21 *panD* cell or other host cell.

The schematic below shows the construction of pSK - MUT, in which four PCR fragments were sequenced and pieced together to form the complete mutase gene in pSK-bluescript.

5



In vivo acyl-CoA analysis in BL21 (DE3) *panD* strains



In follow-up experiments, the specific activity of the mutase was determined and an in-depth CoA analysis was completed. The CoA levels in the cells were again analyzed using a *panD* strain, which is a β -alanine auxotroph. ^3H - β -alanine was fed to the cells and incorporated into the acyl-CoAs, which were separated via HPLC and counted. The CoA pools for cell extracts with and without the mutase, as well as with and without hydroxocobalamin, were examined.

- 10 To test whether acyl-CoAs degrade in TCA, the following tests were conducted. The CoA mix consisted of 1.6 mM each of malonyl-, methylmalonyl-, succinyl-, acetyl-, and propionyl-CoA, plus 0.5 mM CoA. An aliquot (10 μL) of this mix was added to 100 μL 10% TCA, 50 μL were immediately injected to the HPLC for CoA analysis, and the remainder was promptly frozen on dry ice. The frozen portion was then thawed and loaded
- 15

immediately to the HPLC. Again, 10 μ L of the CoA mix were added to 100 μ L 10% TCA, 50 μ L were left on ice for 15 minutes and then injected to the HPLC, the remainder was left at 4°C overnight and injected to the HPLC the next morning. The area under each CoA peak was noted. This same procedure was followed but using a mixture of TCA and buffer A from the mutase assay.

The CoA analysis described here is carried out on cells which are lysed in 10% TCA. Thus, determining whether the CoAs degrade significantly in TCA and in a mixture of TCA and buffer A from the mutase assay is important. The tests showed that the percent of each CoA relative to the total CoA pool, as well as the overall amount of CoA, remained constant after freeze/thawing, after leaving on ice for 15 minutes, and after leaving the sample overnight at 4°C. Thus, the CoAs are stable in TCA and in the mutase assay buffer after the cells are lysed or after the assays are completed, and prior to HPLC analysis.

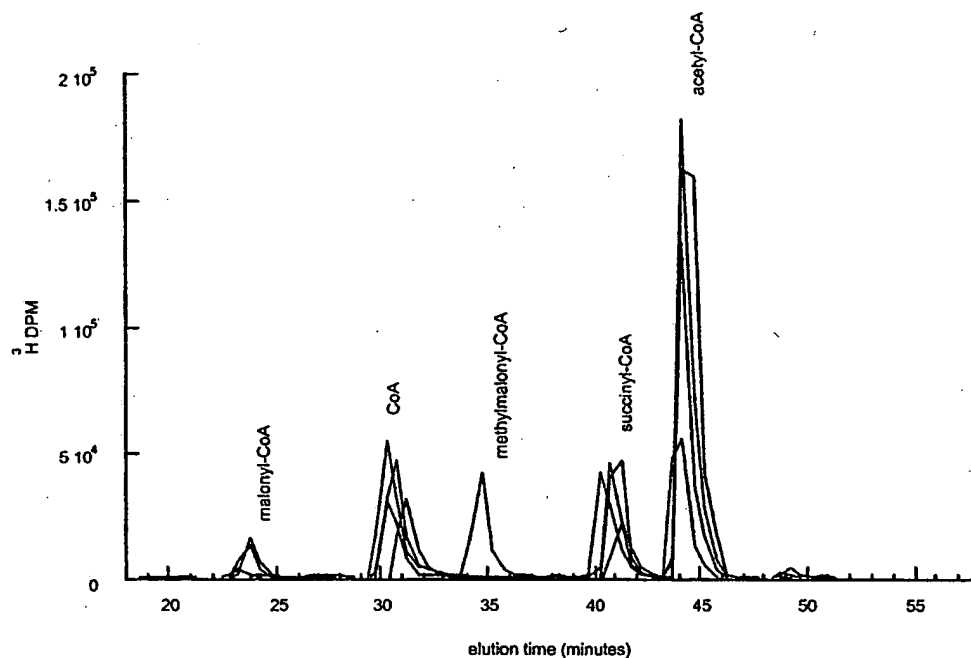
Although the CoAs are stable in TCA and buffer at 4°C, they degraded at 30°C, the temperature at which the mutase assay was performed. In five minutes under the assay conditions, about 4% of the methylmalonyl-CoA hydrolyzed to CoA. The succinyl-CoA hydrolyzed at a comparable rate. Thus, the mutase assay is suboptimal for extremely quantitative results.

When 0.2 mM methylmalonyl-CoA was incubated with a crude lysate from cell extracts overexpressing the mutase, succinyl-CoA was produced. No succinyl-CoA was observed when methylmalonyl-CoA was incubated with lysates from the control strain (containing the plasmid vector but lacking the mutase genes). Under these expression and assay conditions, a specific activity of approximately 0.04 U/mg was observed in the crude extracts. When cells overexpressing the mutase were grown in MUT media without hydroxocobalamin, no mutase activity was observed; however, mutase activity could be detected by addition of vitamin B12 *in vitro*. Adding vitamin B12 to extracts that were grown in the presence of hydroxocobalamin resulted in increased mutase activity, suggesting that a significant amount of

expressed mutase is present as the apo-enzyme. This might have occurred because the enzyme was expressed faster than the hydroxocobalamin could be transported into the cell, or because the vitamin B12 cofactor was lost during preparation of the extract.

5 The graph below shows the comparison of *in vivo* acyl-CoA levels with and without the mutase and with and without hydroxocobalamin. In the cells overexpressing the mutase and grown with hydroxocobalamin, methylmalonyl-CoA comprised 13% of the overall CoA pool, whereas in the other cells no methylmalonyl-CoA was detectable. The background level of
10 counts is about 0.25% of the overall number of counts in the CoAs, suggesting that any methylmalonyl-CoA present in *E. coli* strains not overexpressing the mutase would comprise at most 0.25% of the overall CoA pool, or 2% of the amount of methylmalonyl-CoA observed in the strain overexpressing the mutase. The composition of the CoA pool observed for the *E. coli panD* strain
15 is consistent with that observed previously for *E. coli panD* mutants grown on glucose.

 Thus, the methylmalonyl-CoA mutase from *P. shermanii* has been overexpressed as the active holoenzyme in *E. coli* and shown to produce (2R)-methylmalonyl-CoA *in vivo*. Conversion of (2R)- to (2S)-methylmalonyl-CoA
20 via methylmalonyl-CoA epimerase should provide an adequate supply of the correct isomer of methylmalonyl-CoA to support heterologous production of complex polyketides *E. coli*.



The graph above shows the results of CoA analysis of *E. coli* overexpressing methylmalonyl-CoA mutase. The levels of ^3H detected in fractions collected from HPLC of cell-free extracts from ^3H β -alanine-fed *E. coli* harboring either

5 the pET control vector grown without hydroxocobalamin (black trace), pET grown with hydroxocobalamin (blue trace), pET overexpressing the mutase and grown without hydroxocobalamin (green trace), or pET overexpressing the mutase and grown with hydroxocobalamin (red trace) are shown.

B. Cloning and expression of methylmalonyl-CoA epimerase

The mm-CoA epimerase from *Propionibacterium shermanii* was purified and used to obtain N-terminal protein sequence as well as internal peptide sequence from LysC-generated peptides. The epimerase gene was cloned
5 using hybridization probes designed from the peptide sequences.

Propionibacterium freudenreichii subsp. shermanii was obtained and cultured as described in part A. Purification of mm-CoA epimerase from *P. shermanii*
10 was based on a modification of the published procedure. The procedure utilized a 10 L culture, which was lysed by sonication followed by column chromatography in the order: DE-52, Hydroxyapatite, Phenylsepharose, MonoQ anion exchange, and C-8 RP HPLC.

All operations were performed at 4°C, except the C-8 RP HP2C, which was performed at room temperature, and all buffers contained 0.1 mM PMSF,
15 unless otherwise stated. The epimerase assay was performed essentially as described in the literature. Protein concentration was determined using the method of Bradford. The overall yield of epimerase activity was not determined.

More specifically, cell paste (75 g) was resuspended in 50 mL buffer
20 (50mM Tris-HCl pH 7.5, .1M KCl, 0.2mM PMSF, 1mM EDTA) and sonicated using a macrotip with a diameter of 1.2 cm. With pulses of .5 seconds ON and .3 seconds OFF, the cells were sonicated twice for 30 seconds each at a power setting of 4, followed by five times for 30 seconds each at a power setting of 6. A clear, amber-colored supernatant (53.5 ml) was obtained after spinning for
25 35 minutes at 12,000 rpm.

The crude extract from above was applied to a column (diameter 2.5 cm, height 15 cm) of 73 mL of DE-52 resin equilibrated with 50mM Tris-HCl pH 7.5, .1M KCl. The column was washed at 1 ml/min with three column volumes of the above buffer, followed by a linear gradient to 50mM Tris-HCl
30 pH 7.5, 0.5 M KCl over seven column volumes. Six mL fractions were collected and assayed for epimerase activity. The epimerase was found

predominantly in the flow-through and in several early fractions. The flow-through and active fractions were combined (325 mL) and dialyzed against 4 liters of 50 mM Tris-HCl pH 7.5, 10% glycerol, followed by 4 liters of 10 mM sodium phosphate pH 6.5, 10% glycerol (final volume 250 mL).

- 5 A 7.5 mL hydroxyapatite biogel HTP gel column (diameter 1.5 cm, height 16 cm) was equilibrated with 10 mM sodium phosphate pH 6.5, 5% glycerol. After loading of the enzyme solution (using repeated injections) and washing with three column volumes of the above buffer, a gradient to 200 mM sodium phosphate pH 6.5, 5% glycerol was effected over 20 column
10 volumes at a flow rate of 1 ml/min. The 2 mL fractions were assayed for epimerase activity, and fractions containing epimerase activity were pooled for a total of 99 mL.

- To the 99 mL sample from above, solid ammonium sulfate to 1.5 M final concentration was added slowly and with stirring at 4°C over 30
15 minutes. This suspension (100 mL) was loaded, by repeated injection, onto a 6.6 mL column (1 cm x height 8.5 cm) of phenyl-sepharose resin equilibrated in 20 mM sodium phosphate buffer pH 6.5, 1.5 M ammonium sulfate. The column was washed at 1 ml/min with three column volumes of this buffer, followed by a linear gradient to 20 mM sodium phosphate buffer pH 6.5, 10%
20 glycerol, over 24 column volumes. After assaying the 3 mL fractions for epimerase activity, the fractions containing epimerase activity were pooled and dialyzed against 50mM Tris-HCl pH 7.5.

- A mono Q 5/5 prepacked column was equilibrated with 25 mM Tris-HCl pH 7.5 at 0.5 mL/min. The sample from the previous step was loaded
25 onto the column, which was then washed with 5 column volumes of the above buffer, followed by a linear gradient to 50 mM Tris-HCl pH 7.5, 1 M NaCl, 5% glycerol, over 50 column volumes. The 1 mL fractions were assayed for epimerase activity. Several fractions containing epimerase activity were stored separately; the fraction with the most activity was used for the next
30 purification step.

A reverse-phase column was equilibrated with water containing 0.1% trifluoroacetic acid; 120 μ L (concentrated from 0.5 mL of the active fraction from above, using an Amicon microconcentrator) was injected onto the column at a flow rate of 0.2 mL/min and washed for five minutes with the
5 above solvent system. Then a linear gradient over 50 minutes to acetonitrile containing 0.1% trifluoroacetic acid was implemented. The peaks were collected manually and the peak corresponding to the epimerase (as determined by SDS/PAGE) was dried to completeness, resuspended in water and stored at -80°C .

10 For Lys C mediated digestion of the HPLC-purified epimerase, the epimerase fraction (11751rp2-B, 200 μ L) collected from reverse phase HPLC was dried to completeness and resuspended in 40 μ L water. To 30 μ L of the sample was added 5 μ L of 1 M Tris/HCl, pH 8, 1.5 μ L of .1 M DTT, 2 μ L of Lys C protease (0.2 μ g). A control reaction contained all of the above
15 components except the epimerase. The reactions were incubated overnight at 37°C . An aliquot of the reaction (5 μ L) was diluted to 60 μ L with water and loaded to the HPLC, using the same HPLC program that was used to purify the epimerase. The analytical HPLC showed that the Lys C digestion was not complete. An additional aliquot of Lys C (0.2 μ g) was added to the reactions
20 and incubation was continued overnight at 37°C . Following overnight incubation, an aliquot of the reaction (5 μ L) was diluted to 60 μ L with water and subjected to the HPLC. The HPLC showed that the digestion was complete. The remainder of the reaction was loaded to the HPLC and individual peaks were collected manually. HPLC of the control reaction
25 showed no significant peptide fragments arising from self-digestion of the LysC.

An aliquot of the pure epimerase, as well as a peptide collected from the procedure described above, were submitted for N-terminal amino acid sequencing. Based on the amino acid sequences from above, several
30 degenerate primers were designed as described below that introduced unique restriction sites to either end of the eventual PCR product. These primers

were used in PCR with *P. shermanii* genomic DNA to obtain a 200 base-pair product, which was cloned into a Bluescript™ (Stratagene) vector and submitted for sequencing.

5 A cosmid library of *P. shermanii* was prepared, essentially as described in the Stratagene cosmid manual. The titer of this cosmid library was approximately 11 cfu (colony forming units) per μL , for a total yield of 5556 cfu. A plasmid library of *P. shermanii* was prepared by digesting *P. shermanii* genomic DNA with *SacI* and ligating the resulting mixture into a Bluescript™ vector also cut with *SacI*. To determine the average insert size (2 kb), ten
10 random clones were digested with *SacI*. The ligation mixture was re-transformed 5 times, pooled and plated on one large LB (carb) plate, resulting in a lawn of colonies that were scraped together and resuspended in LB as the plasmid library. The titer of this plasmid library was approximately 64,000 cfu per μL .

15 Several degenerate primers based on the amino acid sequences were prepared and used in PCR with *P. shermanii* genomic DNA to obtain a 180 base-pair product, which was cloned into a Bluescript™ vector and sequenced. Several different probes were made. The first probe was made using the random priming method to incorporate either ^{32}P or digoxigenin
20 into the epimerase fragment. A probe was made from the cloned fragment by amplification of the fragment via PCR, using the digoxigenin labeling method. The PCR product was gel isolated, quantified, and used to probe the cosmid library. Colonies that hybridized to the probe were restreaked from master plates, and five colonies from the re-streaked plates were picked,
25 cosmids were isolated, and the insert sequences screened for the epimerase gene by PCR. Several cosmids that were scored positive for epimerase DNA sequence by PCR were subjected to DNA sequencing using epimerase-specific primers. The cosmid designated 117-167-A7 contained the full epimerase sequence.

30 The sequence of the putative epimerase gene contained in cosmid 117-167-A7 was aligned to the N-terminal epimerase sequence already known.

The several hundred base pairs downstream of this sequence were translated in all three frames and a stop codon in one of the frames was found that yielded a protein of the expected size. The entire sequence was used to search the protein database via BLAST analysis, and the sequence showed high

5 homology to the sequence of a putative epimerase from *S. coelicolor* identified in accordance with the methods of the invention. PCR primers were designed based on the DNA sequence of the cloned *P. shermanii* epimerase and the gene was amplified from *P. shermanii* genomic DNA with *Nde*I and *Bam*HI sites at the 5'-end, an internal *Nde*I site was destroyed near the 5' end, and

10 *Nhe*I and *Avr*II sites were introduced at the 3'-end. Following PCR, the 447 bp product was cloned into a Bluescript vector (143-6-11) and sequenced. Also, four additional sequencing primers were designed to provide several-fold coverage of the epimerase gene. The full epimerase gene sequence provided in isolated and recombinant form by the present invention is shown

15 below.

```

                                     50
ATGAGTAATGAGGATCTTTTCATCTGTATCGATCACGTGGCATATGCGTG
M S N E D L F I C I D H V A Y A C
                                     100
CCCCGACGCCGACGAGGCTTCCAAGTACTACCAGGAGACCTTCGGCTGGC
P D A D E A S K Y Y Q E T F G W
                                     150
ATGAGCTCCACCGCGAGGAGAACCCGAGCAGGGAGTCGTCGAGATCATG
H E L H R E E N P E Q G V V E I M
                                     200
ATGGCCCCGGCTGCGAAGCTGACCGAGCACATGACCCAGGTTCAGGTCAT
M A P A A K L T E H M T Q V Q V M
                                     250
GGCCCCGCTCAACGACGAGTCGACCGTTGCCAAGTGGCTTGCCAAGCACA
A P L N D E S T V A K W L A K H
                                     300
ATGGTCGCGCCGGACTGCACCACATGGCATGGCGTGTGATGACATCGAC
N G R A G L H H M A W R V D D I D
                                     350
GCCGTCAGCGCCACCCTGCGCGAGCGCGCGTGCAGCTGCTGTATGACGA
A V S A T L R E R G V Q L L Y D E
                                     400
GCCCAAGCTCGGCACCGCGGCAACCGCATCAACTTCATGCATCCCCAAGT
P K L G T G G N R I N F M H P K
CGGGCAAGGGCGTGTCTATCGAGCTACCCAGTACCCGAAGAACTGA
S G K G V L I E L T Q Y P K N *

```

The epimerase gene was then cloned into a pET expression vector; the

45 construct was named pET-epsperm.

For the cloning of epimerase genes from *B. subtilis* (described by Haller *et al., supra*) and *S. coelicolor* (from cosmid 8F4 in the *S. coelicolor*, genome sequencing project), primers were designed to PCR these genes from their respective genomic DNAs and to incorporate either a *PacI* or *NdeI* site at the 5' end, and an *NsiI* site at the 3' end. The PCR products were cloned into a Bluescript™ vector and sequenced. Mutation-free clones were obtained for the *S. coelicolor* epimerase, but the *B. subtilis* epimerase contained two point mutations in all three clones tested: C to T at base pair 37, and G to A at base pair 158. When the PCR for this epimerase gene was repeated and the product cloned and sequenced, the same mutations were present, implying that the original sequence was in error. The cloned epimerases from *B. subtilis* and *S. coelicolor* were cloned as *NdeI*/*NsiI* fragments into an intermediate vector 116-172a, a Bluescript™ pET plasmid containing the T-7 promoter and terminator sequences. The cloned epimerases from *B. subtilis* and *S. coelicolor* are pET-epsub and pET-epcoel, respectively. The epimerase genes were also excised along with the T7 promoter as *PacI*/*NsiI* fragments, as shown schematically below.

---*PacI*---T7 promoter-----epimerase gene-----*NsiI*---

and cloned into the *PacI*/*NsiI* restricted vector 133-9b, to form a single operon with the epimerase gene located downstream of the two mutase genes. The epimerase gene from *P. shermanii* was cloned as above except that it was cloned into 116-172a as an *NdeI*/*AvrII* fragment, excised along with the T7 promoter as a *PacI*/*NheI* fragment, and cloned into 133-9b between *PacI* and *NheI* sites. The constructs are pET-mutAB-T7-epsherm, pET-mutAB-T7-epsub, and pET-mutAB-T7-epcoel.

As an alternative to the mutase from *P. shermanii*, *S. coelicolor*, and *B. subtilis*, one can clone by PCR from *E. coli* genomic DNA the single gene for Sbm (sleeping beauty mutase). Genomic DNA of *E. coli* BL21(DE3)/*PanD* was prepared using a kit purchased from Qiagen. The gene for Sbm (Sleeping

beauty mutase, a methylmalonyl-CoA mutase) was amplified by PCR from *E. coli* BL21(DE3)/*PanD* genomic DNA. The PCR fragment was gel isolated, cloned into PCRscript and sequenced to yield the mutation-free clone 143-11-54. Excised as an *NdeI*/*SacI* fragment, *sbm* was cloned into pET22b, thence as a *NdeI*/*XhoI* fragment into pET16b to introduce an N-terminal His-Tag (143-49-2). *Sbm* was also cloned between *NdeI* and *SpeI* into 116-95B.43, a pET22b vector that allows subsequent cloning of the epimerase genes downstream of the *sbm*. That construct was named 143-40-39.

Cells of strain BL21(DE3) containing pET-epsperm, pET-epcoel, pET-epsub, or a control pET vector were grown overnight at 37°C in 2 mL LB containing 100 µg/ml carbenicillin. The starter culture (250 µL) was used to inoculate 25 mL LB containing 100 µg/ml carbenicillin. The cultures were grown at 37°C to an OD of approximately 0.4, then induced with IPTG to 1 mM final concentration and grown for an additional 3 hours at 30°C. The cells were collected by centrifugation at 4000 rpm for 10 minutes, and the pellets were stored at -80°C prior to assay. The epimerase from *P. shermanii* expressed well in *E. coli*; SDS gel analysis revealed an overexpressed protein at approximately 22 kDa. The *S. coelicolor* epimerase also expressed well, at a molecular weight of approximately 19 kDa, and the *B. subtilis* epimerase was expressed, but mostly in inclusion bodies (a faint band is present at approximately 19 kDa), which can be overcome by use of alternate expression systems.

Epimerase activity was measured in crude extracts of *E. coli* harboring either pET-epsperm, pET-epcoel, pET-epsub, or a control pET vector. The epimerase assay couples transcarboxylase, which converts (S)-methylmalonyl-CoA into propionyl-CoA, to malate dehydrogenase, which converts NADH into NAD⁺, producing a decrease in absorbance at 340 nm. The assay is initiated with a racemic mixture of (R,S)-methylmalonyl-CoA; when the (S)-isomer is consumed as described below; a steady background rate is observed at about one-tenth of the initial rate. When an extract containing epimerase is added to the assay, the (R)-isomer is converted to (S)-, resulting in a further decrease in absorbance. In crude *E. coli* extracts, however, a significant

background rate is observed, probably due to an endogenous NADH oxidase. Thus the epimerase must be expressed at a sufficiently high level to conclude that it is active. The assay was conducted as follows.

The pellet from approximately 20 mL of culture was thawed and
5 resuspended in 2 mL 1X assay buffer containing a protease inhibitor cocktail tablet. The cells were disrupted by sonication (two sonication cycles for 30 seconds each at a power setting of 2 [pulse ON 0.5 sec/pulse OFF 0.5 sec]). After spinning for 10 minutes at 13,000 rpm in an Eppendorf centrifuge, the supernatants were saved for assay. Methylmalonyl-CoA epimerase activity
10 was assayed using a modification of the method of Leadlay *et al.* (1981). The assays were performed at 30°C with a 1 cm path length plastic cuvette, in a final volume of 1.5 mL. The reaction mixtures contained 0.2 M potassium phosphate buffer pH 6.9, 0.1 M ammonium sulfate, 5 mM sodium pyruvate, 0.08 mM (2R,2S)-methylmalonyl-CoA, 0.05 units of partially purified
15 transcarboxylase, 0.16 mM NADH, and 2.5 units malate dehydrogenase. The reaction was initiated with (2R,2S)-methylmalonyl-CoA and the decrease in absorbance at 340 nm was monitored, reflecting the disappearance of the 2S isomer. When the decrease in absorbance at 340 nm reached the basal level (usually around 10% of the initial transcarboxylase rate), an extract containing
20 epimerase was added and a further decrease in absorbance was observed. The chemicals and enzymes used in the epimerase assay were purchased from Sigma, except for transcarboxylase, which was obtained as a crude preparation from Case Western Reserve.

The crude extracts harboring both the *P. shermanii* and *S. coelicolor*
25 epimerases had specific activities (approximately 30 units/mg) at least 10 times higher than that of the control. However, no activity above the background level was observed in the extract harboring the *B. subtilis* epimerase, possibly because it was not expressed at a high enough level, or as noted above, was expressed as insoluble inclusion bodies. The pET-mutAB-
30 T7-epsherm construct was also expressed in *E. coli*. The resulting crude extract contained epimerase activity that was significantly above the

background level; thus, the epimerase is functional in this construct. The mutase did not interfere in the epimerase assay, because these cells were grown without addition of hydroxocobalamin, the cofactor for mutase activity. These results show that one can express both active mutase and
5 active epimerase in an *E. coli* cell. These results also show that the methylmalonyl-CoA epimerase from *P. shermanii* was cloned, expressed in *E. coli*, and active, and that the putative epimerase from *S. coelicolor* is a methylmalonyl-CoA epimerase. These genes can be integrated into the chromosome of an *E. coli* PanD strain or other strain and used for the
10 production of polyketides built in whole or in part from methylmalonyl CoA.

Example 2

Production of Methylmalonyl CoA in Yeast

This example describes the construction of strains of *Saccharomyces*
15 *cerevisiae* optimized for polyketide overproduction. In particular, this example describes the construction of yeast host strains that (i) produce substrates and post-translational modification enzymes necessary to express polyketides made by modular polyketide synthases; (ii) have necessary nutritional deficiencies to allow positive selection of at least three compatible
20 plasmids; and/or (iii) are suitable to permit radioactive labeling of acyl-CoA pools and polyketide synthases and demonstrates that such strains can express a modular PKS and produce a complex polyketide at levels suitable for commercial development. References are cited in this example by a number corresponding to the numbered list of references below, each of
25 which is incorporated herein by reference.

With appropriate strain modifications, *S. cerevisiae* is an ideal host for polyketide production. *S. cerevisiae* is capable of producing very high levels of polyketides. Introduction of the gene for the iterative PKS, 6-MSAS, along with the gene for Sfp, a P-pant transferase from *B. subtilis*, led to the
30 production of an impressive 2 g/L 6-MSA in shake-flasks without optimization [3]. The genetics of yeast is very well understood. Genes can

readily be inserted into the chromosome, and the complete genome sequence provides relevant knowledge regarding metabolic pathways and neutral insertion sites. In addition, several strong, controllable promoters are available. Proteins have less tendency to form inclusion bodies in yeast, compared to *E. coli*. Yeast has a relatively short doubling time in comparison to native polyketide producing organisms. *S. cerevisiae* has a doubling time of 1 to 2 hr compared to 4 to 24 hr for a typical polyketide producer, which has obvious benefits in genetic development, process development, and large-scale production.

10 The fact that yeast grow as single cells provides an additional benefit over filamentous organisms (typical polyketide producers). Mycelial fermentations are viscous and frequently behave as non-Newtonian fluids. This fluid rheology provides a significant obstacle to the process scientist both in terms of uniform nutrient transport to the cells and in handling the fermentation broth. Employing yeast as a host, even at high cell densities, avoids such impediments. Because of the extensive history of yeast in single cell protein production and the expression of recombinant proteins, scalable fermentation protocols for yeast have been developed. Yeast can be grown in fed-batch fermentations to very high cell densities (>100 g/L biomass) as compared to typical polyketide producers (10-20 g/L biomass). Thus, comparing organisms with the same specific productivity (g polyketide/g biomass/day), yeast would provide a higher volumetric productivity (g polyketide/L/day). Finally, *S. cerevisiae* is classified by the FDA as a "Generally Regarded As Safe" (GRAS) organism. GRAS classification will facilitate approval of drugs produced in yeast as compared to other host cells.

25 *S. cerevisiae* also has *disadvantages* as a host for polyketide biosynthesis, most of which are related to the fact that yeast did not evolve to produce polyketides. Yeast does not contain methylmalonyl-CoA, a necessary precursor for biosynthesis of many polyketides. Yeast does not have a suitable P-pant transferase capable of the necessary post translational modification of ACP domains of a PKS. Yeast codons are biased towards A+T,

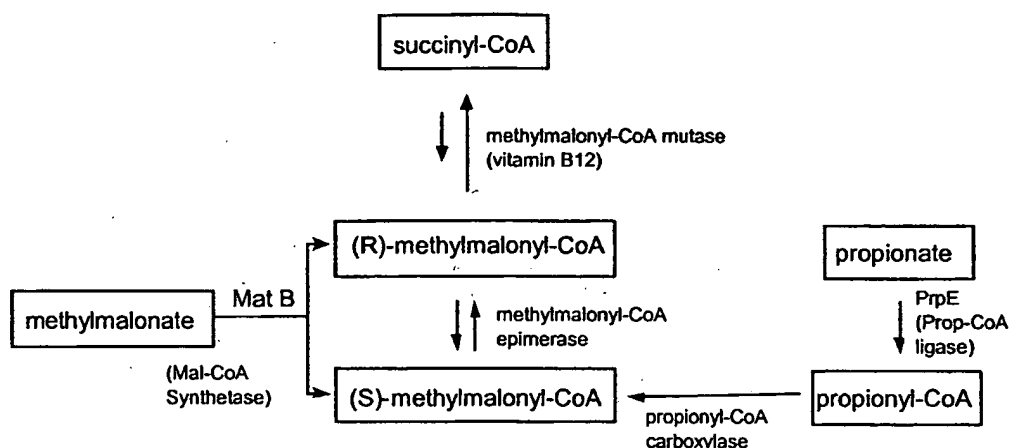
whereas most polyketide producers have high G+C codons; thus, yeast may have low amounts of some tRNAs needed for PKS gene expression. The correction of these deficiencies is described in this example, and the invention also provides modified yeast host cells useful to facilitate analysis of success.

5 Other case-by-case potential issues with yeast include the possibility that some polyketide products may be toxic or may require additional modifications for maturation (e.g. glycosylation, P450 hydroxylation). Several methods provided by the invention may be taken to circumvent these issues should they arise. For toxicity, production may be controlled to occur in
10 stationary phase growth (as with 6-MSA production); resistance factors from the wild type host may be introduced into the yeast host (e.g. methylation of ribosomes for some antibiotics); a non-toxic-precursor to the polyketide may be produced and converted *ex vivo* (e.g. produce 6-dEB in one strain and convert it to erythromycin in another), and others. Additional modifications
15 to the polyketide may be accomplished by cloning and expressing modification enzymes into the host strain, chemical or enzymic transformation, and/or biosynthetic transformation in a second strain (e.g. convert 6-dEB analogs to erythromycin analogs by feeding 6-dEB to a *Streptomyces* or *Saccharopolyspora* strain capable of glycosylation and P450
20 hydroxylation).

 Most modular PKSs require either or both malonyl-CoA or (2S)-methylmalonyl-CoA as a source of 2-carbon units for polyketide biosynthesis. The malonyl-CoA pools in yeast are quite sufficient for polyketide synthesis, as illustrated the production of large amounts of 6-MSA in yeast. However, *S.*
25 *cerevisiae* does not produce (2S)-methylmalonyl-CoA and does not possess biosynthetic pathways for methylmalonyl-CoA biosynthesis. Hence, a heterologous biosynthetic pathway must be introduced into *S. cerevisiae* to support biosynthesis of polyketides that use (2S)-methylmalonyl-CoA as a precursor.

30 There are three routes or biosynthetic pathways for the synthesis of methylmalonyl-CoA that can be engineered into yeast, as shown in the

schematic below. These pathways have been shown to produce methylmalonyl-CoA in *E. coli* and can be used to produce methylmalonyl-CoA in yeast. This example describes the identification of a system for methylmalonyl-CoA production in yeast, and a method for introducing it into the yeast chromosome.



Biosynthetic pathways to (S)-methylmalonyl-CoA

The vitamin B12-dependent methylmalonyl-CoA mutase pathway produces (2R)-methylmalonyl-CoA from succinyl-CoA. The (2R)-methylmalonyl-CoA is converted to the (2S)- diastereomer via methylmalonyl-CoA epimerase, as shown above. These enzymes are present in a variety of organisms, but not yeast; BLAST searches of the available genomic databases reveals at least 10 methylmalonyl-CoA mutases and 10 methylmalonyl-CoA epimerases in various organisms. The *Propionibacterium shermanii* methylmalonyl-CoA mutase has been expressed in *E. coli* as the apo-enzyme, which requires addition of vitamin B12 for *in vitro* activity [4]. By use of a medium that enables uptake of the vitamin B12 precursor hydroxocobalamin [5], and in accordance with the methods of the invention, one can express active *P. shermanii* methylmalonyl-CoA mutase holoenzyme in *E. coli* and produce (2R)-methylmalonyl-CoA in such cells. In addition, one can employ the single subunit methylmalonyl-CoA mutase from *E. coli*. The

present invention also provides the genes encoding methylmalonyl-CoA epimerase from *B. subtilis*, *P. shermanii* and *S. coelicolor* and methods for using them in converting (2*R*)-methylmalonyl-CoA to the needed (2*S*)-diastereomer. A preferred method is to express in yeast the methylmalonyl-CoA mutase from *E. coli*, because it is a single ORF, and necessary codons are plentiful in yeast. Alternatively, the *P. shermanii* enzyme can be used.

PCC catalyzes the biotin-dependent carboxylation of propionyl-CoA to produce (2*S*)-methylmalonyl-CoA, as shown above; the pathway also includes a biotin carrier protein/biotin carboxylase. In *S. coelicolor*, Rodriguez and Gramajo identified genes for PCC (*pccB*) and a biotin carrier protein/biotin carboxylase (*accA1*) [6]. Introduction into *E. coli* of *S. coelicolor* *pccB* and *accA1* along with propionyl-CoA ligase (as a supply of propionyl-CoA), results in the production of methylmalonyl-CoA in that organism. A search of the genomic database reveals *B. subtilis* as an additional source of the enzymes involved in the PCC pathway.

In one embodiment of the invention, one can express the *S. coelicolor* *pccB* and *accA1* in yeast, because these are expressed and the proteins are functional in *E. coli*. Should codon usage prove suboptimal when expressing the *S. coelicolor* genes in yeast, homologs from *B. subtilis* can be employed. Should the levels of propionyl-CoA be suboptimal for PCC, one can co-express a propionyl-CoA ligase in the yeast host. Intracellular propionyl-CoA can be greatly increased in *E. coli* by expressing the *Salmonella* propionyl-CoA ligase, PrpE, and supplementing the growth media with propionate, as described below.

An additional method for the production of (2*S*)-methylmalonyl-CoA provided by the present invention utilizes the *matB* and *matC* genes from *Rhizobium* [7] or *S. coelicolor* (see schematic above). The *matABC* genes code for a biosynthetic pathway that converts malonate to acetyl-CoA through formation of malonyl-CoA via MatB and subsequent decarboxylation by MatA. MatB, the malonyl-CoA ligase, also accepts methylmalonate as a substrate [7] and catalyzes formation of methylmalonyl-CoA. The substrates

malonate or methylmalonate enter the cell through a diacid transporter, the product of the *matC* gene. Khosla *et al.* have shown that when *E. coli* containing the *Rhizobium matBC* is fed (2R,2S)-methylmalonate, (2R,2S)-methylmalonyl-CoA is produced. Furthermore, when an *S. coelicolor* strain
5 expressing the genes for the synthesis of the polyketide aglycone, 6-deoxyeythronolide B (6-dEB), and containing *Rhizobium matBC*, is fed methylmalonate, a 3-fold increase in production of 6-dEB is observed. In accordance with the methods of the invention, one can express the *matB* and *matC* genes from *Rhizobium* in yeast, because these are expressed and the
10 proteins are functional in *E. coli* and *S. coelicolor*, or, alternatively the *matBC* genes from *S. coelicolor*.

Active PKSs require post-translational phosphopantetheinylation at each ACP of each module, but yeast does not contain a P-pant transferase with the needed specificity [3]. Previous work [3] has shown that
15 introduction of the *B. subtilis* P-pant transferase gene, *sfp*, into yeast results in an expressed Sfp capable of modifying an iterative PKS, 6-MSAS. Gokhale *et al.* demonstrated that the ACP domains in the DEBS PKS are substrates for Sfp, so Sfp is a general modifying enzyme for PKSs [8]. In preferred yeast host cells of the invention, the *sfp* gene is inserted into a neutral site of the
20 yeast chromosome.

In developing a system to produce polyketides and optimize fermentation procedures, the ability to measure intracellular concentrations of substrates (i.e. acyl-CoAs) and of the PKS is beneficial. In most cells, CoA esters are not present in sufficient amounts to allow direct measurement by
25 HPLC using ultraviolet detection or other simple methods of detection. In *E. coli*, the method of choice to quantify CoA pools is to feed [³H] β-alanine to a mutant deficient in aspartate decarboxylase (*PanD*), which cannot produce endogenous β-alanine [9]. The *PanD* strain incorporates about ten-fold more radioactivity into CoA pools than does wild type *E. coli*. Because β-alanine is
30 a direct precursor of CoA, the radioactive label enters the CoA pool without dilution, and acyl-CoAs can be separated on HPLC and quantified by

radioactivity measurement. Because there is no radioisotope dilution, the radioactivity measured reflects exact intracellular concentrations of the acyl-CoAs.

- BLAST searches did not reveal an *E. coli* *PanD* homolog in the yeast genome; however, yeast may be a β -alanine or pantothenate auxotroph. Indeed, for CoA biosynthesis, yeast requires either exogenous pantothenate, which enters the cell via the Fen2p transporter, or exogenous β -alanine, which enters via the general amino acid permease (Gap1p) [10]. [3 H] β -alanine is incorporated into CoA pools of yeast (see below), but it is presently unknown whether isotope dilution occurs due to endogenous β -alanine production by some unknown pathway. Thus, to enable quantitation, one can determine the specific activity of CoA pools in yeast labeled with exogenous [3 H] β -alanine. Cells producing polyketides generally express low levels of high molecular weight PKSs that are barely detectable on SDS-PAGE using protein stains.
- The ability to label CoA with [3 H] β -alanine can also be used to quantify a PKS expressed in the host cells because the phosphopantetheine moiety of CoA containing β -alanine is transferred to the ACP domain in each module of a PKS. Thus, knowing the specific activity of labeled intracellular CoAs, a PKS can be simply quantified by radioactivity after SDS-PAGE.
- The G+C content of most PKS genes is in the range of 60 to 70%, while that of yeast genes is 40%. Thus, some tRNAs needed to translate PKS genes are scarce (but not absent) in yeast. However, many genes with high G+C content have been expressed in yeast. As examples, the large (1560 bp) DHFR-TS gene from *Leishmania major* (63% G+C) is expressed well in yeast, despite the fact that it contains several codons rarely used in yeast [11]. Moreover, as mentioned below, the PKS 6-MSAS (G+C = 58%) is also expressed well in yeast [3]. Thus, one can demonstrate the general applicability of a yeast expression system without initial concern for potential codon usage problems. Nevertheless, if a desired PKS does not express well in yeast, the present invention provides several methods to solve a "codon usage" problem observed with a particular polyketide.

First, one can change the codons at the 5' end of the gene to reflect those more frequently found in yeast genes. Batard *et al.* [12] successfully employed a similar method to express in yeast wheat genes for a P450 and P450 reductase with high G+C content (56%) and strong bias of codon usage unfavorable to yeast. Another method is to introduce yeast tRNA genes with anti-codons modified to represent codons common in PKS sequences. A similar method has been successfully used in *E. coli* to enhance expression of high G+C genes [13], including PKS genes from Actinomycetes. A third method is to synthesize chemically the gene with codons optimized for expression in yeast. The cost for contract synthesis of a 30,000 bp gene (e.g. ~6-module PKS), including sequence verification, is approximately \$3 per base, or about \$100,000. For a valuable product (e.g. epothilone), the cost is not prohibitive.

In an illustrative embodiment of the invention, a yeast strain deficient in Ura, Trp, His and Leu biosynthesis is employed as a host to allow selection of plasmids containing these markers. This host is modified in accordance with the methods of the invention by introducing genes that produce the needed methylmalonyl-CoA substrate and P-pant transferase for post-translational modifications of PKSs. These are preferably integrated into the yeast chromosome, because they are necessary for production of any polyketide. To validate functional expression of the substrate genes, one can measure methylmalonyl-CoA pools. For validation of P-pant transferase activity, one can coexpress 6-MSAS and measure [³H] phosphopantetheinylation of the enzyme as well as 6-MSA production. Should either be deficient, one can increase gene copy number.

For PKS gene expression, one can use replicating vectors based on the 2 micron replicon, because plasmids may have to be rescued for analysis should a problem arise. A typical modular PKS gene cluster (e.g. 3 ORFs, ~10 kB each, as in erythromycin) can be introduced on three or more vectors; such plasmids (containing Ura, Trp and Leu markers) are available and similar to those used in the studies of 6-MSAS expression in yeast. A PKS consisting of

three large proteins can be functionally reconstituted from separately expressed genes [14]. Once a system is established for a particular PKS of interest, one can integrate the PKS genes into stable, neutral sites of the chromosome.

5 Preferred promoters include the glucose repressible alcohol dehydrogenase 2 (ADH2) promoter and the galactose-inducible (GAL1) promoter. The former has been used to produce high amounts of the polyketide 6-MSA in yeast, and the latter is highly controllable by galactose in the medium.

10 A model modular PKS that one can use to optimize the yeast host is the well studied DEBS1. In this model system, the first ORF of the modular PKS for erythromycin biosynthesis (DEBS1) has been fused to a thioesterase domain (TE) and produces a readily detectable triketide lactone when expressed in *S. coelicolor*, and more recently *E. coli* [20] [21]. The gene
15 contains 2 PKS modules, is about 12 kDa, and produces a protein that is 300 kDa. This model allows one to optimize the engineered host for acyl-CoA levels and post-translational modifications, the PKS for G+C content, and to develop the needed analytical methods. Once optimized for DEBS1, one can express any given modular PKS.

20 Previously, it has been shown that the fungal gene encoding 6-methylsalicylic acid synthase (6-MSAS) from *Penicillium patulum* was expressed in *S. cerevisiae* and *E. coli* and the polyketide 6-methylsalicylic acid (6-MSA) was produced [3]. In both bacterial and yeast hosts, polyketide production required co-expression of 6-MSAS and a heterologous
25 phosphopantetheinyl transferase (Sfp), which was required to convert the expressed apo-PKS to the holo-enzyme. Production of 6-MSA in *E. coli* was both temperature- and glycerol-dependent and levels of production (~60 mg/L) were lower than those of the native host, *P. patulum*. In yeast, the 6-MSAS and *sfp* genes were co-expressed from separate replicating plasmids,
30 and gene expression was driven by the glucose repressible alcohol dehydrogenase 2 (ADH2) promoter. In a non-optimized shake flask

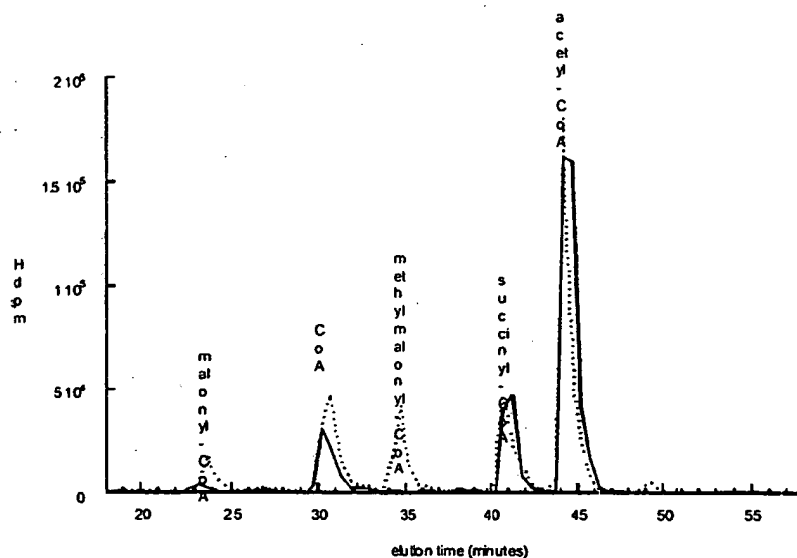
fermentation, the yeast system produced 6-MSA at levels of 2,000 mg/L. This was the first report of expression of an intact PKS gene in yeast or *E. coli*, and demonstrated that extraordinarily high levels of polyketides can be produced in yeast.

5 Previously, a two vector system was developed for heterologous expression of the three genes comprising the DEBS polyketide gene cluster [15]. Individual DEBS genes and pairwise combinations of two such genes were each cloned downstream of the actinorhodin (*actI*) promoter in two compatible *Streptomyces* vectors: the autonomously replicating vector, 10 pKAO127'Kan', and the integrating vector, pSET152. When the resulting plasmids were either simultaneously or sequentially transformed into the heterologous host, *Streptomyces lividans* K4-114, the polyketide product, 6-dEB, was produced. This work showed that the DEBS genes could be split apart and expressed on separate plasmids, and that efficient *trans*- 15 complementation of modular polyketide synthase subunit proteins occurred in the heterologous host.

 A three-plasmid system for heterologous expression of DEBS has been developed to facilitate combinatorial biosynthesis of polyketides made by type I modular PKSs [14]. The *eryA* PKS genes encoding the three DEBS 20 subunits were individually cloned into three compatible *Streptomyces* vectors carrying mutually selectable antibiotic resistance markers. A strain of *Streptomyces lividans* transformed with all three plasmids produced 6-dEB at a level similar to that of a strain transformed with a single plasmid containing all three genes. The utility of this system in combinatorial biosynthesis was 25 demonstrated through production of a large library of greater than 60 modified polyketide macrolactones, using versions of each plasmid constructed to contain defined mutations. Combinations of these vector sets were introduced into *S. lividans*, resulting in strains producing a wide range of 6-dEB analogs. This method can be extended to any modular PKS and has the 30 potential to produce thousands of novel natural products, including ones derived from further modification of the PKS products by tailoring enzymes.

Moreover, the ability to express the modular PKSs (such as DEBS) from three separate plasmids provides advantages in the commercialization of polyketide production by heterologous expression of modular PKSs in yeast and *E. coli* in accordance with the methods of the present invention.

- 5 As described in Example 1, the translationally coupled genes, *mutA* and *mutB*, encoding the β - and α -subunits of methylmalonyl-CoA mutase from *Propionibacterium shermanii*, were amplified by PCR and inserted into an *E. coli* expression vector containing a T-7 promoter. The naturally occurring GTG start codon for *mutB* was changed to ATG to facilitate expression [5].
- 10 Heterologous expression of the mutase genes in media containing [^3H] β -alanine and the adenosylcobalamin (coenzyme B₁₂) precursor, hydroxocobalamin, yielded active methylmalonyl-CoA mutase. HPLC analysis of extracts from *E. coli* BL21(DE3)/*panD* harboring the mutase genes indicated production of methylmalonyl-CoA, which comprised 13% of the
- 15 intracellular CoA pool (shown below). This work demonstrates that one can introduce a biosynthetic pathway for an important PKS substrate into a heterologous host, and that one can measure the intracellular concentration of acyl-CoAs. In accordance with the present invention, the methylmalonyl-CoA mutase gene (*sbm*) from *E. coli*, which has codon usage closer to yeast
- 20 and encodes a single polypeptide [16], can also be employed.

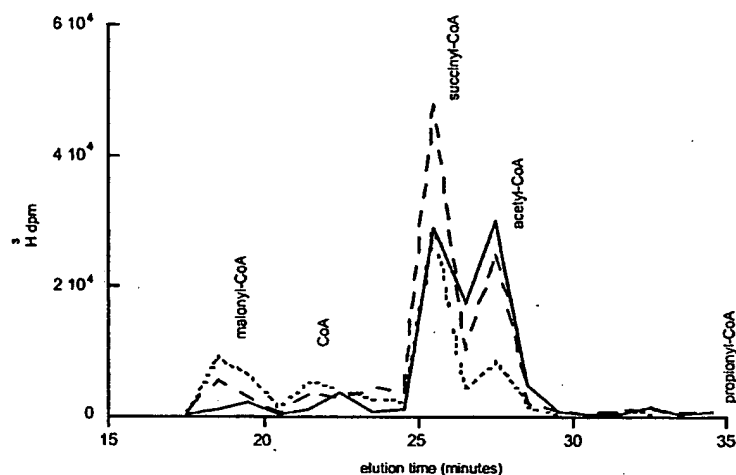


The graph above shows acyl-CoA analysis of *E. coli* overexpressing methylmalonyl-CoA mutase. The level of ^3H detected in fractions collected from HPLC of cell-free extracts from [^3H] β -alanine-fed *E. coli* harboring either the pET control vector (solid trace) or pET overexpressing the mutase (dashed trace) is shown.

As described in Example 1, methylmalonyl-CoA epimerase was purified from *Propionibacterium shermanii* and N-terminal and internal protein sequence was obtained. Degenerate PCR primers based on the amino acid sequences were designed and were used to amplify a 180 bp PCR product from *P. shermanii* genomic DNA. The PCR product was labeled and used to isolate the epimerase gene from a *P. shermanii*. The methylmalonyl-CoA epimerase genes from *B. subtilis* [16] and *S. coelicolor* can also be employed in the methods of the present invention.

Propionyl-CoA is not detected in *E. coli* SJ16 cells grown in the presence of [^3H] β -alanine with or without the addition of propionate in the growth media. When *E. coli* SJ16 cells were transformed with a pACYC-derived plasmid containing the *Salmonella typhimurium* propionyl-CoA ligase gene (*prpE*) under the control of the *lac* promoter, a small amount of propionyl-CoA was observed (~0.2% of total CoA pool) in cell extracts. When

5 mM sodium propionate was included in the culture medium, about 14-fold more propionyl-CoA was produced (~ 3% of the total CoA pool). These results are shown graphically below.



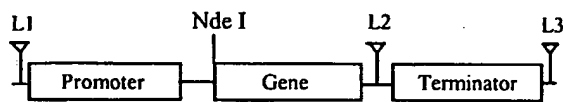
5

The graph above shows acyl-CoA analysis in *S. cerevisiae*. The level of ^3H detected in fractions collected from HPLC of cell-free extracts from [^3H] β -alanine-fed *S. cerevisiae* after growth for 24 hours (solid trace), 48 hours (dashed trace) and 66 hours (dotted trace) is shown. The yeast strain InvSc1 [3], grown in synthetic YNB media lacking pantothenate and β -alanine, was used for acyl-CoA analysis. Yeast cultures starved of β -alanine were fed [^3H] β -alanine and the cultures were grown for 24, 48 and 66 hours at 30°C. Cells were disrupted with glass beads in the presence of 10% cold TCA and acyl-CoAs were separated by HPLC and quantified by scintillation counting. The yeast CoA pools were labeled with [^3H], but the extent of isotope dilution remains unclear. One can measure the specific activity of total CoA in these strains to ascertain the extent of isotope dilution.

For PKS genes and initial studies of metabolic pathway genes, one can employ the analogous sets of bluescript cloning vectors and yeast 2 micron replicating shuttle vectors used in 6-MSA production [3]. With these vectors, yeast expression is driven by the alcohol dehydrogenase 2 (ADH2) promoter,

which is tightly repressed by glucose and is highly active following glucose depletion that occurs after the culture reaches high density. Both vector sets have a "common cloning cassette" that contains, from 5' to 3', a polylinker (L1), the ADH2 (or other) promoter, a *Nde* I restriction site, a polylinker (L2), an ADH2 (or other) terminator, and a polylinker (L3). Due to excess restriction sites in the yeast shuttle vectors, genes of interest are first introduced into intermediate bluescript cloning vectors via the *Nde* I site, to generate the ATG start codon, and a downstream restriction site in the L2 polylinker that is common to the bluescript and yeast shuttle vectors (shown below). The promoter-gene cassette is then excised as an L1-L2 fragment and transferred to the yeast expression vector containing the transcriptional terminator.

Common Cloning Cassette



Host strains for model systems include commonly available yeast strains with nutritional deficiencies (Ura, Trp, His, Leu) that can harbor at least three replicating vectors (see below). If it is necessary to express more than three PKS genes simultaneously, one can clone multiple promoter-PKS gene-terminator cassettes into the same vector or use a fourth replicating vector with a different nutritional marker (i.e. Leu) or an antibiotic marker (i.e. G418). One can also construct an analogous set of bluescript cloning and yeast expression/shuttle vectors containing a galactose-inducible promoter. The galactose promoter-Gal4 activator system is more tightly regulated than the ADH2 promoter, and may be beneficial or necessary for expression of proteins that are toxic to yeast [17].

Genes involved in the production of substrates (e.g. methylmalonyl-CoA and/or propionyl-CoA), and the *sfp* gene can preferably be stably integrated into the yeast chromosome in appropriate copy number to produce adequate levels of desired acyl-CoAs and post translational PKS

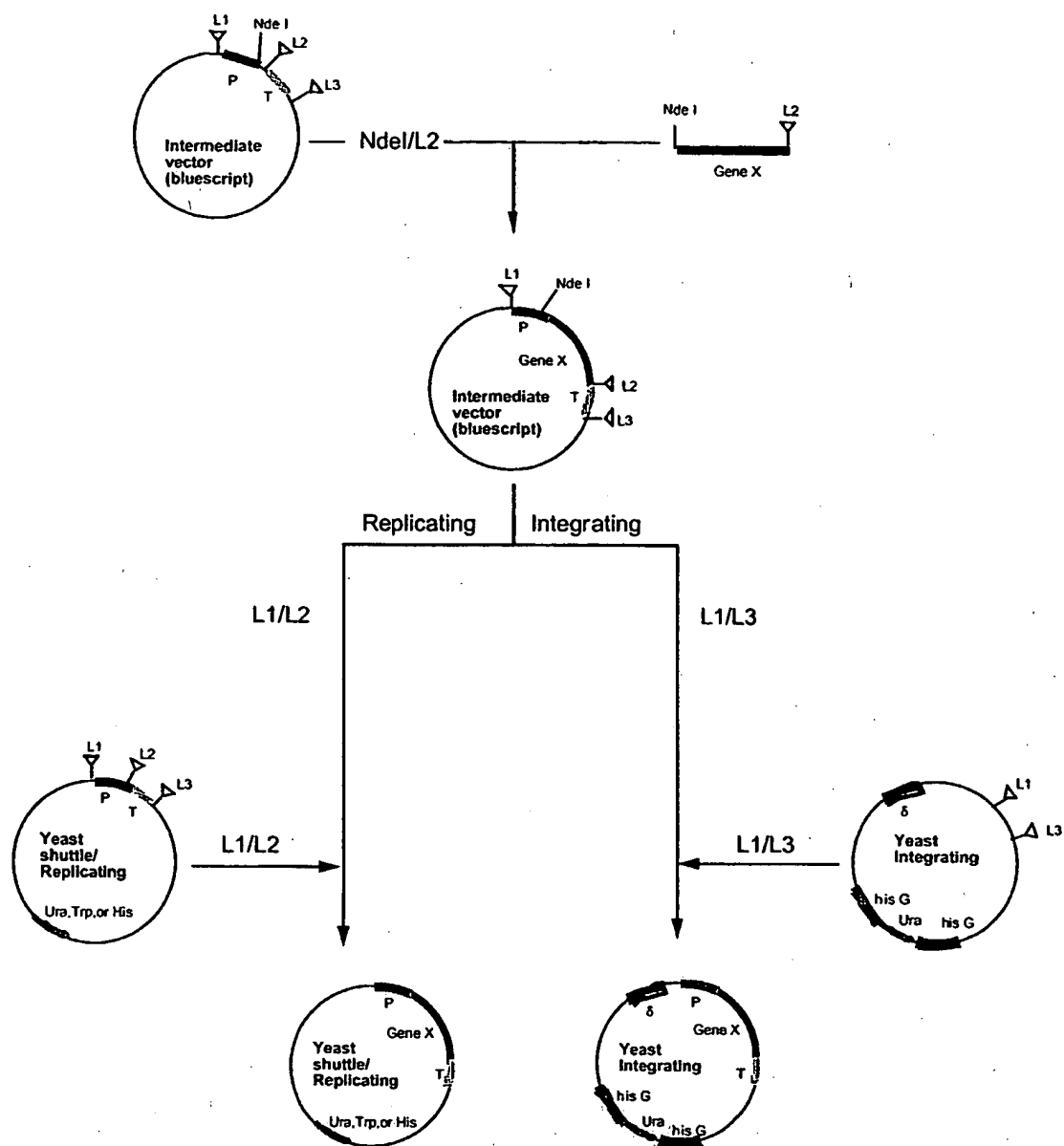
modifications. Genes can first be introduced into the intermediate bluescript cloning vector as described. Then, the fragment containing the promoter-gene-terminator cassette can be transferred as a L1-L3 fragment to a yeast "delta integration" vector [18] [19] that allows chromosomal integration of the cassettes into one or more of the ca. 425 delta sequences dispersed throughout the yeast chromosome (see the schematic below). These vectors have cloning sites compatible with those in the L1-L3 linkers to permit direct transfer of promoter-gene-terminator cassettes as L1-L3 fragments. They also contain the excisable Ura3 selection marker flanked by two bacterial *hisG* repeats ("URA Blaster"), enabling insertion of multiple identical or different genes into the yeast chromosome by repetitive integrations. After selection for gene integration on media lacking uracil, the Ura3 gene fragment is removed by selecting for marker loss via excisional recombination by positive selection with 5-fluoroorotic acid (FOA), which renders the Ura3 gene toxic to yeast. This enables the introduction of stable pathways needed for acyl-CoA precursors and Sfp into yeast, while conserving the Ura marker to allow its subsequent use in plasmids containing other genes.

The single-gene mutase, Sbm (Sleeping beauty mutase), from *E. coli* [16], can be cloned as follows. Primers designed based on the DNA sequence were used to PCR amplify the *sbm* gene from *E. coli* genomic DNA as a *NdeI*-L2 fragment. The general strategy for cloning the genes into yeast expression vectors follows that of Kealey *et al.* [3] (see the schematic below). One can first clone the genes as *NdeI*-L2 fragments into the intermediate bluescript cloning vector. The promoter-gene-terminator cassette can then be excised as an L1-L3 fragment, transferred to the yeast integrating vector, restricted with L1/L3, and introduced into the yeast chromosome as described above. As an alternative to Sbm, one can use the two-gene mutase from *P. shermanii*; the translationally coupled genes have each been amplified by PCR as *NdeI*-L2 fragments and can be integrated into yeast as described above.

The genes encoding matABC have been cloned into a bluescript vector [7]. One can isolate the matB (methylmalonyl-CoA ligase) and matC

(dicarboxylic acid transporter) genes by PCR, each as a *Nde*I-L2 fragment, and integrate them into the yeast chromosome as described above and shown in the schematic below. Yeast transformed with *matBC* will be treated with methylmalonic acid, and cells extracts can be analyzed for methylmalonyl-CoA.

The *pccB* and *accA1* genes involved in the propionyl-CoA carboxylation pathway in *S. coelicolor* can be amplified by PCR from genomic DNA. As shown in the schematic below, the genes can be cloned into the intermediate bluescript vector between *Nde* I and L2, then transferred to the yeast integrating vector via L1/L3. One can express the *S. coelicolor* genes shown to be effective in *E. coli*; should codon usage be suboptimal, one can employ the *B. subtilis* orthologs (discussed above).



The schematic above shows a general method for cloning genes into yeast expression vectors.

In one embodiment, the recombinant yeast host cells of the invention co-express the *B. subtilis* P-pant transferase, *Sfp*, with a PKS to convert the apo PKS to its holo form. The *sfp* gene is available on Bluescript™

(Stratagene) cloning and yeast shuttle/expression vectors and is functional in yeast [3], so one can simply construct stable strains expressing this gene. One to several copies (as determined optimal) of the *sfp* gene can be introduced into delta sequences in the yeast chromosome as described above. One can
5 test the activity of the integrated *sfp* gene by co-expressing 6-MSAS on a replicating vector, by measuring the Sfp-dependent 6-MSA production [3], and by quantifying the incorporation of [^3H] β -alanine into the ACP domain of the PKS (see below). This allows one to determine the optimal number of copies of the *sfp* gene needed for maximal polyketide production.

10 The gene for the modular PKS, DEBS1+TE, is available as a *NdeI-EcoRI* fragment, which can be readily introduced into a yeast shuttle/expression vector as indicated in the schematic above. Yeast strains expressing DEBS1+TE are analyzed for the [^3H]-phosphopantetheinylation of the PKS, and for production of triketide lactone by liquid chromatography/mass
15 spectrometry.

^3H labeling of intracellular Acyl-CoAs is carried out as follows. Cells are treated with [^3H] β -alanine (available at 50 Ci/mmol) in defined media lacking pantothenate, enabling the radioactive precursor of pantothenate to enter the CoA pool. Cells are then disrupted, CoA esters are separated by
20 HPLC, and the radioactivity quantified by liquid scintillation counting, as described above.

Saccharomyces cerevisiae host cells are grown, and extracts prepared as follows. Defined minimal YNB media (1 mL) lacking pantothenate but containing 1 μM β -alanine are inoculated with a single colony of *S. cerevisiae*
25 (InvSc1, or Fen2b deletion strain) from a YPD plate. The culture is grown to stationary phase and 10 μl of the stationary culture are used to inoculate the above media lacking β -alanine and pantothenate. The culture is incubated for 4 hours and 10 μl of the "starved" culture is used to inoculate media (1 mL) containing 10 μCi [^3H] β -alanine (50 Ci/mmol; 0.2 μM final β -alanine). After
30 culture growth for appropriate times, the cells from a 1 mL culture are collected by centrifugation and washed with water. The cells are suspended

in 200 μ l of 10% cold trichloroacetic acid (TCA), containing standard unlabeled acyl-CoAs as chromatography markers (malonyl-, methylmalonyl-, succinyl-, acetyl-, propionyl-CoA, and CoA). The cells are disrupted by vortexing with glass beads, and the supernatant analyzed by HPLC.

5 HPLC is performed using a 150 x 4.6mm 5 μ ODS-3 INERTSIL HPLC column purchased from Metachem technology. HPLC buffer A is 100mM sodium phosphate monobasic, 75 mM sodium acetate, pH 4.6 and buffer B is 70% buffer A, 30% methanol. The HPLC column is equilibrated at 10% buffer B at a flow rate of 1 mL/min. Following injection, a linear gradient to 40%
10 buffer B is implemented over 35 minutes, followed by a linear gradient to 90% buffer B over 20 minutes. The gradient affords base-line separation of the standard acyl-CoAs. The eluant is monitored at 260 nm and fractions are collected and counted in a scintillation counter.

Determination of the specific activity of the total CoA pool is carried
15 out as follows. *S. cerevisiae* cultures are labeled with 100 μ Ci of [3 H] β -alanine as described above. The yeast cells are disrupted and the extract is treated with 100 μ M hydroxylamine, pH 8.5, to convert all acyl-CoAs to CoA. The labeled CoA is isolated by HPLC as described above and converted to acetyl-CoA with *E. coli* acetyl-CoA synthase (Sigma), using [14 C]-acetate as a
20 substrate. The [3 H, 14 C]-acetyl-CoA is separated by HPLC and the dual labels quantified by scintillation counting. The mmol CoA is determined by 14 C, and specific activity of CoA determined from the 3 H dpm per mmol CoA. The isotope dilution, reflecting endogenous production of β -alanine, is calculated by the specific activity of [3 H] CoA/ specific activity [3 H] β -alanine
25 used in the test.

Analysis of PKS expression levels is carried out as follows. Each ACP domain of each module of an active PKS is post-translationally modified with phosphopantetheine derived from CoA. Using yeast cells treated with [3 H] β -alanine (described above), one can label the PKS with high specific activity
30 tritium. The protein will be separated on SDS-PAGE, eluted and radioactivity determined by liquid scintillation counting.

The references cited in the preceding example are listed below, and each is incorporated herein by reference.

1. Crosby, J., et al., *Polyketide synthase acyl carrier proteins from Streptomyces: expression in Escherichia coli, purification and partial characterisation.* Biochim Biophys Acta, 1995. 1251(1): p. 32-42.
2. Roberts, G.A., J. Staunton, and P.F. Leadlay, *Heterologous expression in Escherichia coli of an intact multienzyme component of the erythromycin-producing polyketide synthase.* Eur J Biochem, 1993. 214(1): p. 305-11.
3. Kealey, J.T., et al., *Production of a polyketide natural product in nonpolyketide-producing prokaryotic and eukaryotic hosts.* Proc Natl Acad Sci U S A, 1998. 95(2): p. 505-9.
4. McKie, N., et al., *Adenosylcobalamin-dependent methylmalonyl-CoA mutase from Propionibacterium shermanii. Active holoenzyme produced from Escherichia coli.* Biochem J, 1990. 269(2): p. 293-8.
5. Amaratunga, M., et al., *A synthetic module for the metH gene permits facile mutagenesis of the cobalamin-binding region of Escherichia coli methionine synthase: initial characterization of seven mutant proteins.* Biochemistry, 1996. 35(7): p. 2453-63.
6. Rodriguez, E. and H. Gramajo, *Genetic and biochemical characterization of the alpha and beta components of a propionyl-CoA carboxylase complex of Streptomyces coelicolor A3(2).* Microbiology, 1999. 145(Pt 11): p. 3109-19.
7. An, J.H. and Y.S. Kim, *A gene cluster encoding malonyl-CoA decarboxylase (MatA), malonyl-CoA synthetase (MatB) and a putative dicarboxylate carrier protein (MatC) in Rhizobium trifolii—cloning, sequencing, and expression of the enzymes in Escherichia coli.* Eur J Biochem, 1998. 257(2): p. 395-402.
8. Gokhale, R.S., et al., *Dissecting and exploiting intermodular communication in polyketide synthases.* Science, 1999. 284(5413): p. 482-5.
9. Jackowski, S. and C.O. Rock, *Regulation of coenzyme A biosynthesis.* J Bacteriol, 1981. 148(3): p. 926-32.

10. Stolz, J. and N. Sauer, *The fenpropimorph resistance gene FEN2 from Saccharomyces cerevisiae encodes a plasma membrane H⁺-pantothenate symporter*. J Biol Chem, 1999. 274(26): p. 18747-52.
11. Grumont, R., W. Sirawaraporn, and D.V. Santi, *Heterologous expression of the bifunctional thymidylate synthase-dihydrofolate reductase from Leishmania major*. Biochemistry, 1988. 27(10): p. 3776-84.
12. Batard, Y., et al., *Increasing expression of P450 and P450-reductase proteins from monocots in heterologous systems [In Process Citation]*. Arch Biochem Biophys, 2000. 379(1): p. 161-9.
13. Carstens, C.-P., et al., *New BL21-CodonPlus™ Cells Correct Codon Bias in GC-Rich Genomes*. Strategies Newsletter from Stratagene Corp., 2000. 13(1): p. 31-33.
14. Xue, Q., et al., *A multiplasmid approach to preparing large libraries of polyketides*. Proc Natl Acad Sci U S A, 1999. 96(21): p. 11740-5.
15. 15. Ziermann, R., Betlach, M., *A Two-vector System for the Production of Recombinant Polyketides in Streptomyces*. J. Bacter., 1998.
16. Haller, T., et al., *Discovering new enzymes and metabolic pathways: conversion of succinate to propionate by Escherichia coli*. Biochemistry, 2000. 39(16): p. 4622-9.
17. Mylin, L.M., et al., *Regulated GAL4 expression cassette providing controllable and high-level output from high-copy galactose promoters in yeast*. Methods Enzymol, 1990. 185: p. 297-308.
18. Lee, F.W. and N.A. Da Silva, *Improved efficiency and stability of multiple cloned gene insertions at the delta sequences of Saccharomyces cerevisiae*. Appl Microbiol Biotechnol, 1997. 48(3): p. 339-45.
19. Lee, F.W. and N.A. Da Silva, *Sequential delta-integration for the regulated insertion of cloned genes in Saccharomyces cerevisiae*. Biotechnol Prog, 1997. 13(4): p. 368-73.
20. Kao, C.M., et al., *Engineered biosynthesis of a triketide lactone from an incomplete modular polyketide synthase*. J. Am. Chem. Soc., 1994. 116(25): p. 11612-11613.

21. Cortes, J., et al., *Repositioning of a domain in a modular polyketide synthase to promote specific chain cleavage*. Science, 1995. 268(5216): p. 1487-9.

Example 3

5 Conversion of Erythronolides to Erythromycins

A sample of a polyketide (~50 to 100 mg) is dissolved in 0.6 mL of ethanol and diluted to 3 mL with sterile water. This solution is used to overlay a three day old culture of *Saccharopolyspora erythraea* WHM34 (an *eryA* mutant) grown on a 100 mm R2YE agar plate at 30°C. After drying, the plate
10 is incubated at 30°C for four days. The agar is chopped and then extracted three times with 100 mL portions of 1% triethylamine in ethyl acetate. The extracts are combined and evaporated. The crude product is purified by preparative HPLC (C-18 reversed phase, water-acetonitrile gradient containing 1% acetic acid). Fractions are analyzed by mass spectrometry, and
15 those containing pure compound are pooled, neutralized with triethylamine, and evaporated to a syrup. The syrup is dissolved in water and extracted three times with equal volumes of ethyl acetate. The organic extracts are combined, washed once with saturated aqueous NaHCO₃, dried over Na₂SO₄, filtered, and evaporated to yield ~0.15 mg of product. The product is a
20 glycosylated and hydroxylated compound corresponding to erythromycin A, B, C, and D but differing therefrom as the compound provided differed from 6-dEB.

Example 4

25 Measurement of Antibacterial Activity

Antibacterial activity is determined using either disk diffusion assays with *Bacillus cereus* as the test organism or by measurement of minimum inhibitory concentrations (MIC) in liquid culture against sensitive and resistant strains of *Staphylococcus pneumoniae*.

Example 5Evaluation of Antiparasitic Activity

Compounds can initially screened *in vitro* using cultures of *P. falciparum* FCR-3 and K1 strains, then *in vivo* using mice infected with *P. berghei*.

- 5 Mammalian cell toxicity can be determined in FM3A or KB cells. Compounds can also be screened for activity against *P. berhei*. Compounds are also tested in animal studies and clinical trials to test the antiparasitic activity broadly (antimalarial, trypanosomiasis and Leishmaniasis).

- 10 The invention having now been described by way of written description and example, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments and that the foregoing description and examples are for purposes of illustration and not limitation of the following claims.

Claims

1. A recombinant host cell comprising one or more expression vectors that drive expression of enzymes capable of making a product and a precursor required for biosynthesis of the product in said host cell, wherein said host cell, in the absence of said expression vectors, is unable to make said product due to lacking all or a part of a biosynthetic pathway required to produce the precursor.
2. A recombinant host cell comprising one or more expression vectors that drive expression of enzymes capable of making a product and a precursor required for biosynthesis of the product in said host cell, wherein said host cell, in the absence of said expression vectors for said enzymes capable of making said precursor, makes said product in substantially lesser amounts due to said precursor being present in said host in limiting amounts.
3. The host cell of Claim 1 or 2, wherein said precursor is a primary metabolite that is produced in a first cell but not in a second heterologous cell.
4. The host cell of any of Claims 1 or 2, wherein said product is a polyketide.
5. The host cell of Claim 4, wherein said polyketide is a polyketide synthesized by either a modular, iterative, or fungal PKS.
6. The host cell of Claim 5, wherein said precursor is selected from the group consisting of malonyl CoA, propionyl CoA, methylmalonyl CoA, ethylmalonyl CoA, and hydroxymalonyl CoA.

7. The host cell of Claim 6, wherein said precursor is methylmalonyl CoA.

8. The host cell of Claim 7 that is either a procaryotic or eukaryotic
5 host cell.

9. The host cell of Claim 8 that is an *E. coli* host cell.

10. The host cell of Claim 8 that is a yeast host cell.

10

11. The host cell of Claim 8 that is a plant host cell.

12. The host cell of Claim 9, wherein said polyketide is synthesized
by a modular PKS.

15

13. The host cell of Claim 12, wherein said precursor biosynthetic
enzyme is a methylmalonyl CoA mutase that converts succinyl CoA to
methylmalonyl CoA.

14. The host cell of Claim 13, wherein said methylmalonyl CoA
mutase is derived from propionibacteria.

20

15. The host cell of Claim 14, which has been further modified to
overexpress a B12 transporter gene.

25

16. The host cell of Claim 15, wherein said B12 transporter gene is
endogenous to *E. coli*.

17. The host cell of Claim 14 in media that facilitates B12 uptake.

30

18. The host cell of Claim 13 that further comprises an epimerase that converts R-methylmalonyl CoA to S-methylmalonyl CoA.

19. The host cell of Claim 18, wherein said epimerase is derived
5 from propionibacteria.

20. The host cell of Claim 18, wherein said epimerase is derived from *Streptomyces*.

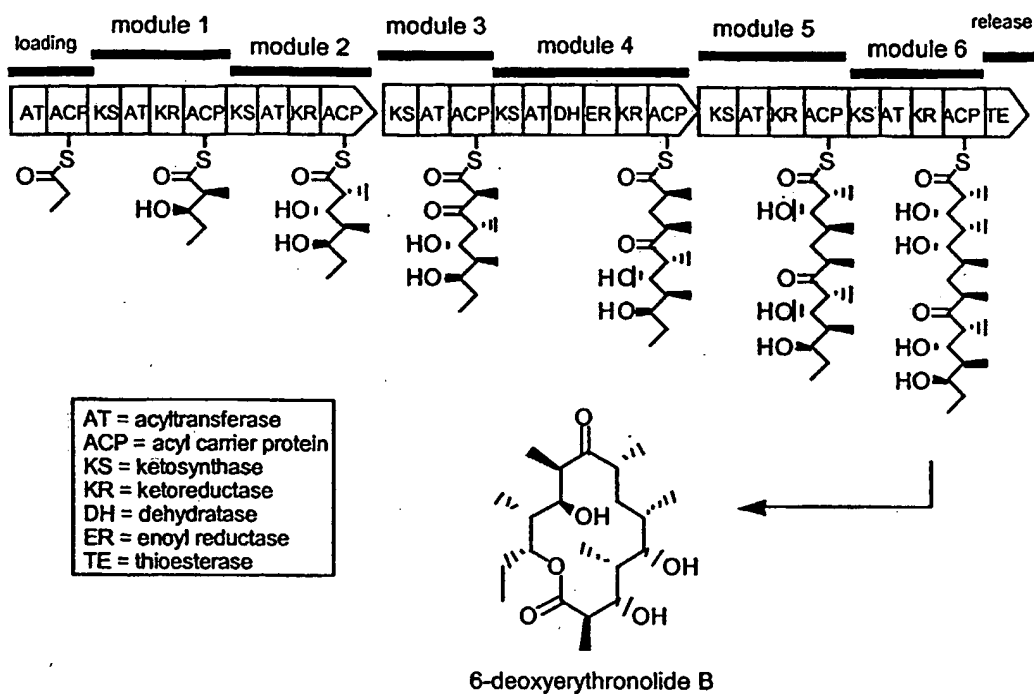
10 21. The host cell of Claim 12, wherein said precursor biosynthetic enzyme is a propionyl CoA carboxylase that converts propionyl CoA to methylmalonyl CoA.

22. The host cell of Claim 21 that has been further modified to
15 overexpress a biotin transferase enzyme.

23. The host cell of Claim 22, wherein said biotin transferase enzyme is encoded by the *birA* gene.

20 24. An *E. coli* host cell that expresses heterologous methylmalonyl CoA mutase and epimerase genes.

25. A yeast host cell that expresses heterologous methylmalonyl CoA mutase and epimerase genes.



Biosynthesis of 6-Deoxyerythronolide B (6-dEB), the Aglycone of Erythromycin, by a Modular PKS

Figure 1

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
3 May 2001 (03.05.2001)

PCT

(10) International Publication Number
WO 01/31035 A3

(51) International Patent Classification⁷: C12N 15/61,
1/21, C12P 11/00, 19/62, C12N 9/90 // (C12N 1/21, C12R
1:19) (C12P 11/00, C12R 1:19) (C12P 19/62, C12R 1:19)

(74) Agents: FAVORITO, Carolyn, A. et al.; Morrison & Foerster LLP, 3811 Valley Centre Drive, Suite 500, San Diego, CA 92130-2332 (US).

(21) International Application Number: PCT/US00/29775

(22) International Filing Date: 27 October 2000 (27.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/161,703 27 October 1999 (27.10.1999) US

(71) Applicant: KOSAN BIOSCIENCES, INC. [US/US];
3832 Bay Center Place, Hayward, CA 94545 (US).

(72) Inventors: SANTI, Daniel; 211 Belgrave Avenue, San Francisco, CA 94117 (US). PECK, Larry; 910 Laurel Street, San Carlos, CA 94070 (US). DAYEM, Linda; 2240 Village Court, #2, Belmont, CA 94002 (US). KEALEY, James; 19 Watt Avenue, San Rafael, CA 94901 (US).

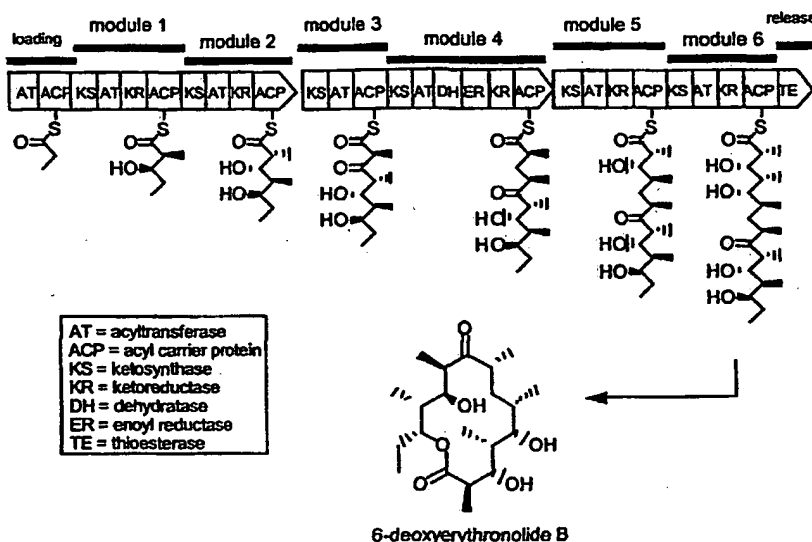
(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— with international search report

[Continued on next page]

(54) Title: HETEROLOGOUS PRODUCTION OF POLYKETIDES



Biosynthesis of 6-Deoxyerythronolide B (6-dEB), the Aglycone of Erythromycin, by a Modular PKS

(57) Abstract: Recombinant host cells that comprise recombinant DNA expression vectors that drive expression of a product and a precursor for biosynthesis of that product can be used to produce useful products such as polyketides in host cells that do not naturally produce the product or produce the product or precursor at low levels due to the absence of the precursor or the presence of the precursor in rate limiting amounts.



(88) Date of publication of the international search report:
22 November 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

Int'l Application No
PC1/US 00/29775

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/61 C12N1/21 C12P11/00 C12P19/62 C12N9/90
/(C12N1/21,C12R1:19),(C12P11/00,C12R1:19),(C12P19/62,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STASSI D L ET AL.: "Ethyl-substituted erythromycin derivatives produced by directed metabolic engineering" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 95, no. 13, 23 June 1998 (1998-06-23), pages 7305-7309, XP002131683 ISSN: 0027-8424 abstract page 7308, left-hand column, line 4 -right-hand column, line 17 --- -/--	1-6

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

11 July 2001

Date of mailing of the international search report

24/07/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

van de Kamp, M

INTERNATIONAL SEARCH REPORT

Int'l Application No
PC1/US 00/29775

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TANG L ET AL.: "Amino acid catabolism and antibiotic synthesis: valine is a source of precursors for macrolide biosynthesis in Streptomyces ambofaciens and Streptomyces fradiae" JOURNAL OF BACTERIOLOGY, vol. 176, no. 19, October 1994 (1994-10), pages 6107-6119, XP001009878 abstract page 6116, line 18-65 figure 8	2-8
A	--- KAO C M ET AL.: "Engineered biosynthesis of a complete macrolactone in a heterologous host" SCIENCE, vol. 265, no. 5171, 22 July 1994 (1994-07-22), pages 509-512, XP002073974 ISSN: 0036-8075 abstract page 510, left-hand column, line 64 -right-hand column, line 18 page 511, left-hand column, line 12 -middle column, line 9	1-13,18, 21,24,25
A	--- ROBERTS G A ET AL.: "Heterologous expression in Escherichia coli of an intact multienzyme component of the erythromycin-producing polyketide synthase" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 214, no. 1, 15 March 1993 (1993-03-15), pages 305-311, XP001009958 cited in the application abstract	1-9,12
A	--- MCKIE N ET AL.: "Adenosylcobalamin-dependent methylmalonyl-CoA mutase from Propionibacterium shermanii - Active holoenzyme produced from Escherichia coli" BIOCHEMICAL JOURNAL, vol. 269, no. 2, 15 July 1990 (1990-07-15), pages 293-298, XP001010015 abstract --- -/--	13,14, 24,25

INTERNATIONAL SEARCH REPORT

Int tional Application No
PC 1/US 00/29775

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>VRIJBLOED J W ET AL.: "Insertional inactivation of methylmalonyl coenzyme A (CoA) mutase and isobutyryl-CoA mutase genes in Streptomyces cinnamonensis: Influence on polyketide antibiotic biosynthesis." JOURNAL OF BACTERIOLOGY, vol. 181, no. 18, 1999, pages 5600-5605, XP002171679 ISSN: 0021-9193 abstract</p>	13
A	<p>LEADLAY P F: "Purification and characterization of methylmalonyl-CoA epimerase from Propionibacterium shermanii" BIOCHEMICAL JOURNAL, vol. 197, no. 2, 1981, pages 413-420, XP000100996 ISSN: 0264-6021 cited in the application abstract</p>	18,19, 24,25
A	<p>BRAMWELL H ET AL.: "Propionyl-CoA carboxylase from Streptomyces coelicolor A3(2): Cloning of the gene encoding the biotin-containing subunit." MICROBIOLOGY (READING), vol. 142, no. 3, 1996, pages 649-655, XP001009712 ISSN: 1350-0872 abstract page 650, left-hand column, line 10-23 page 653, left-hand column, line 7 -right-hand column, line 4</p>	21
A	<p>DONADIO S ET AL.: "Erythromycin production in Saccharopolyspora erythraea does not require a functional propionyl-CoA carboxylase." MOLECULAR MICROBIOLOGY, vol. 19, no. 5, 1996, pages 977-984, XP001009721 ISSN: 0950-382X abstract page 977, left-hand column, line 1 -right-hand column, line 41 page 982, right-hand column, line 27-47</p>	21
A	<p>WO 99 19518 A (DIVERSA INC ;SHORT JAY M (US)) 22 April 1999 (1999-04-22) page 4, line 12 -page 6, line 19 page 11, line 22 -page 12, line 25 page 24, line 1-6 page 25, line 7-20</p>	1-12
	-/--	

INTERNATIONAL SEARCH REPORT

Int tional Application No
PC1/US 00/29775

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 27203 A (KOSAN BIOSCIENCES) 25 June 1998 (1998-06-25) page 2, line 29 -page 4, line 28 examples 1-12 claims 1-39 ---	1-12
A	KHOSLA C ET AL: "Tolerance and specificity of polyketide synthases" ANNUAL REVIEW OF BIOCHEMISTRY, vol. 68, 1999, pages 219-253, XP000884453 page 221, line 35-37 page 222, line 5-8 ---	1-6
P,X	WO 00 20601 A (KOSAN BIOSCIENCES INC ;CHU DANIEL (US); KHOSLA CHAITAN (US); SANTI) 13 April 2000 (2000-04-13) page 6, line 29 -page 7, line 11 page 65, line 21 -page 66, line 8 page 67, line 30 -page 70, line 2 claim 17 ---	1-6
P,X	ZHANG W ET AL.: "Molecular analysis and heterologous expression of the gene encoding methylmalonyl-coenzyme A mutase from rifamycin SV-producing strain Amycolatopsis mediterranei U32" APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY, vol. 82, no. 3, December 1999 (1999-12), pages 209-225, XP001009874 abstract page 222, line 12 -page 223, line 2; figure 6; table 1 ---	1-8
P,A	RODRIGUEZ E ET AL.: "Genetic and biochemical characterization of the alpha and beta components of a propionyl-CoA carboxylase complex of Streptomyces coelicolor A3(2)." MICROBIOLOGY (READING), vol. 145, no. 11, November 1999 (1999-11), pages 3109-3119, XP001009722 ISSN: 1350-0872 cited in the application abstract ---	13,24,25
P,A	PFEIFER B A ET AL.: "Biosynthesis of complex polyketides in a metabolically engineered strain of E. coli" SCIENCE, vol. 291, no. 5509, 2 March 2001 (2001-03-02), pages 1790-1792, XP002170306 the whole document page 1790, right-hand column, line 54 -page 1791, right-hand column, line 8 ---	21
T	---	1-9, 21-23
	-/--	

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/29775

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>PFEIFER B A ET AL.: "Biosynthesis of polyketides in heterologous hosts"</p> <p>MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS,</p> <p>vol. 65, no. 1, March 2001 (2001-03), pages 106-118, XP000997626.</p> <p>the whole document</p> <p>page 108, left-hand column, line 13 -page 111, left-hand column, line 8</p> <p>page 113, left-hand column, line 3 -page 116, left-hand column, line 8</p> <p>-----</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/29775

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9919518 A	22-04-1999	AU 1094699 A EP 1029076 A	03-05-1999 23-08-2000
WO 9827203 A	25-06-1998	AU 734325 B AU 5701098 A EP 0948613 A US 6033883 A	07-06-2001 15-07-1998 13-10-1999 07-03-2000
WO 0020601 A	13-04-2000	AU 1441500 A	26-04-2000

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
19 April 2001 (19.04.2001)

PCT

(10) International Publication Number
WO 01/27306 A3

(51) International Patent Classification⁷: C12P 11/00,
19/62, C12N 1/21 // (C12N 1/21, C12R 1:19) (C12P
11/00, C12R 1:19) (C12P 19/62, C12R 1:19)

(21) International Application Number: PCT/US00/28573

(22) International Filing Date: 13 October 2000 (13.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/159,090 13 October 1999 (13.10.1999) US
60/206,082 18 May 2000 (18.05.2000) US
60/232,379 14 September 2000 (14.09.2000) US

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— with international search report

(71) Applicant: THE BOARD OF TRUSTEES OF THE LE-
LAND STANFORD JUNIOR UNIVERSITY [US/US];
900 Welch Road, Suite 350, Palo Alto, CA 94304 (US).

(72) Inventors: KHOSLA, Chaitan; 211 Belgrave Avenue,
San Francisco, CA 94117 (US). PFEIFER, Blaine; 1450
Oak Creek Drive, #402, Palo Alto, CA 94304 (US).

(74) Agents: MURASHIGE, Kate, H. et al.; Morrison & Foer-
ster LLP, 3811 Valley Centre Drive, Suite 500, San Diego,
CA 92130-2332 (US).

(88) Date of publication of the international search report:
21 February 2002

*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

WO 01/27306 A3

(54) Title: BIOSYNTHESIS OF POLYKETIDE SYNTHASE SUBSTRATES

(57) Abstract: The use of enzymes which catalyze the production of starter and extender units for polyketides is described. These enzymes may be used to enhance the yield of polyketides that are natively produced or polyketides that are rationally designed. By using these techniques, the synthesis of a complete polyketide has been achieved in *E. coli*. This achievement permits a host organism with desirable characteristics to be used in the production of such polyketides and to assess the results of gene shuffling.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/28573

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12P11/00 C12P19/62 C12N1/21 //(C12N1/21, C12R1:19),(C12P11/00,C12R1:19),(C12P19/62,C12R1:19)		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12P C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STASSI D L ET AL.: "Ethyl-substituted erythromycin derivatives produced by directed metabolic engineering" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 95, no. 13, 23 June 1998 (1998-06-23), pages 7305-7309, XP002131683 ISSN: 0027-8424 abstract page 7308, left-hand column, line 4 -right-hand column, line 17 --- -/--	1,4,9, 10,13, 24,30,33
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.		
<input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search 10 July 2001		Date of mailing of the international search report 22.10.01
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer van de Kamp, M

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/28573

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TANG L ET AL.: "Amino acid catabolism and antibiotic synthesis: valine is a source of precursors for macrolide biosynthesis in Streptomyces ambofaciens and Streptomyces fradiae" JOURNAL OF BACTERIOLOGY, vol. 176, no. 19, October 1994 (1994-10), pages 6107-6119, XP001009878 abstract page 6107, left-hand column, line 1 -right-hand column, line 24 page 6116, left-hand column, line 18-65 ---	1,2,4,9, 10,13, 24,30
X	WO 98 27203 A (KOSAN BIOSCIENCES) 25 June 1998 (1998-06-25) page 2, line 29 -page 4, line 28 ---	42
A	examples 1-12 claims 1-39 ---	1-10,12, 13, 24-30,43
X	KAO C M ET AL.: "Engineered biosynthesis of a complete macrolactone in a heterologous host" SCIENCE, vol. 265, no. 5171, 22 July 1994 (1994-07-22), pages 509-512, XP002073974 ISSN: 0036-8075 abstract ---	33-35
A	page 510, left-hand column, line 65 -right-hand column, line 8 ---	1,2,4,6, 9,14,15, 24-26
A	HSIEH Y-J ET AL.: "Inhibition of erythromycin synthesis by disruption of malonyl-coenzyme A decarboxylase gene eryM in Saccharopolyspora erythraea" JOURNAL OF BACTERIOLOGY, vol. 176, no. 3, February 1994 (1994-02), pages 714-724, XP001009880 ISSN: 0021-9193 page 722, left-hand column, line 43-51 ---	1,10,13, 24,30
A	KHOSLA C ET AL.: "Tolerance and specificity of polyketide synthases" ANNUAL REVIEW OF BIOCHEMISTRY, vol. 68, 1999, pages 219-253, XP000884453 page 222, line 35-37 page 222, line 5-8 ---	1,10,13, 24,30
	-/--	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/28573

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>QUADRI L E ET AL.: "Characterization of Sfp, a Bacillus subtilis phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases" BIOCHEMISTRY, vol. 37, no. 6, 10 February 1998 (1998-02-10), pages 1585-1595, XP002170303 abstract</p>	8,12
A	<p>--- UMEYAMA T ET AL.: "Expression of the Streptomyces coelicolor A3(2) ptpA gene encoding a phosphotyrosine protein phosphatase leads to overproduction of secondary metabolites in S. lividans" FEMS MICROBIOLOGY LETTERS, vol. 144, no. 2-3, 1 November 1996 (1996-11-01), pages 177-184, XP001009805 abstract</p>	1,2,4, 10,13, 24,25,30
A	<p>--- DONADIO S ET AL: "Erythromycin production in Saccharopolyspora erythraea does not require a functional propionyl-CoA carboxylase." MOLECULAR MICROBIOLOGY, vol. 19, no. 5, 1996, pages 977-984, XP001009721 ISSN: 0950-382X abstract page 982, right-hand column, line 27-47</p>	12,14, 15,34,35
A	<p>--- ZIERMANN R ET AL.: "Recombinant polyketide synthesis in Streptomyces: engineering of improved host strains" BIOTECHNIQUES, vol. 26, no. 1, January 1999 (1999-01), pages 106-110, XP002170304 abstract</p>	1,2,4,6, 7,9, 24-26
A	<p>--- WO 95 08548 A (JOHN INNES CENTRE ;UNIV LELAND STANFORD JUNIOR (US)) 30 March 1995 (1995-03-30) page 4, line 26 -page 7, line 12 page 21, line 32 -page 31, line 15 examples 1-3 claims 1-30</p> <p>--- -/--</p>	1,2,4,7, 24-26

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/28573

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 99 19518 A (DIVERSA INC ;SHORT JAY M (US)) 22 April 1999 (1999-04-22)</p> <p>page 4, line 12 -page 6, line 19 page 11, line 22 -page 12, line 25 page 24, line 1-6 page 25, line 7-20</p> <p>---</p>	<p>1-5,7,8, 10,11, 13, 24-28, 30,33, 40,42</p>
A	<p>WO 99 03986 A (KOSAN BIOSCIENCES INC) 28 January 1999 (1999-01-28)</p> <p>page 2, line 7 -page 3, line 15 page 4, line 7-28 examples 1-3</p> <p>---</p>	<p>24, 30-33, 40,41</p>
P,X	<p>WO 00 20601 A (KOSAN BIOSCIENCES INC ;CHU DANIEL (US); KHOSLA CHAITAN (US); SANTI) 13 April 2000 (2000-04-13)</p> <p>page 6, line 29 -page 7, line 11 page 67, line 30 -page 70, line 2 claim 17</p> <p>---</p>	<p>1,2,10, 13, 24-26, 30,33</p>
P,X	<p>ZHANG W ET AL.: "Molecular analysis and heterologous expression of the gene encoding methylmalonyl-coenzyme A mutase from rifamycin SV-producing strain Amycolatopsis mediterranei U32" APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY, vol. 82, no. 3, December 1999 (1999-12), pages 209-225, XP001009874 abstract page 222, line 12 -page 223, line 2; figure 6; table 1</p> <p>---</p>	<p>1,2,4, 10,13, 24,25,30</p>
P,A	<p>RODRIGUEZ E ET AL.: "Genetic and biochemical characterization of the alpha and beta components of a propionyl-CoA carboxylase complex of Streptomyces coelicolor A3(2)." MICROBIOLOGY (READING), vol. 145, no. 11, November 1999 (1999-11), pages 3109-3119, XP001009722 ISSN: 1350-0872 cited in the application abstract</p> <p>---</p>	<p>12,14, 15,34,35</p>
	<p>---</p> <p>-/--</p>	

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/28573

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>PFEIFER B A ET AL.: "Biosynthesis of complex polyketides in a metabolically engineered strain of E. coli"</p> <p>SCIENCE, vol. 291, no. 5509, 2 March 2001 (2001-03-02), pages 1790-1792, XP002170306 the whole document</p> <p>---</p>	<p>1-10, 13-15, 24-30, 33-35, 42,43</p>
T	<p>PFEIFER B A ET AL.: "Biosynthesis of polyketides in heterologous hosts"</p> <p>MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, vol. 65, no. 1, March 2001 (2001-03), pages 106-118, XP000997626 the whole document page 108, left-hand column, line 13 -page 111, left-hand column, line 8 page 113, left-hand column, line 3 -page 116, left-hand column, line 8</p> <p>-----</p>	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/28573

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-13, 24-33, 40-43 (all partially); 14, 15, 34, 35 (all compl.)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-13,24-33,40-43 (all partially); 14,15,34, 35 (all completely)

Prokaryotic host cells (e.g., of the genus *Escherichia* (e.g., *E. coli*), *Streptomyces*, *Bacillus*, *Pseudomonas* or *Flavobacterium*) which are genetically modified for enhanced synthesis of at least one (complete) polyketide (e.g., 6-dEB), wherein said modification comprises incorporation of an expression system for producing a functional propionyl-CoA carboxylase that catalyzes the production of starter and/or extender units.

Said host cells which do not produce polyketide in the absence of genetic modification, and wherein said modification comprises incorporation of an expression system for a polyketide synthase (PKS) and incorporation of an expression system for a phosphopantetheinyl transferase. A method to produce a polyketide comprising culturing said cells.

A method to assess the results of a procedure effecting modification of PKS genes using said *E. coli* cells which contain a functional propionyl-CoA carboxylase and possibly further a functional phosphopantetheinyl transferase.

A method to enhance the production of a polyketide in said microbial host cells which method comprises the provision of said host cell with an expression system for propionyl-CoA carboxylase, e.g., encoded by the *pccB* and *accA2* genes from *S. coelicolor*.

Recombinant microbial cells (e.g., *Streptomyces* (*coelicolor* CH999) or *Escherichia* (*coli*)) that produce a (complete) polyketide (e.g., 6-dEB) which have been modified to contain an expression system for (a) nucleotide sequence(s) encoding said propionyl-CoA carboxylase, and methods for producing a polyketide using said cells.

A reaction mixture for the production of a polyketide comprising in addition to enzymes catalyzing the production of said polyketide, said functional propionyl-CoA carboxylase, e.g., encoded by the *pccB* and *accA2* genes from *S. coelicolor*, and methods for producing a polyketide using said reaction mixture.

2. Claims: 1-12,42,43 (all partially)

Prokaryotic host cells (e.g., of the genus *Escherichia* (e.g., *E. coli*), *Streptomyces*, *Bacillus*, *Pseudomonas* or *Flavobacterium*) which are genetically modified for enhanced synthesis of at least one (complete) polyketide (e.g., 6-dEB), wherein said modification comprises deleting a pathway responsible for propionate catabolism, e.g., the *prpA-D* operon of *E. coli*.

Said host cells which do not produce polyketide in the

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

absence of genetic modification, and wherein said modification comprises incorporation of an expression system for a polyketide synthase (PKS) and incorporation of an expression system for a phosphopantetheinyl transferase. A method to produce a polyketide comprising culturing said cells.

A method to assess the results of a procedure effecting modification of PKS genes using said *E. coli* cells which contain a functional phosphopantetheinyl transferase and which have been further modified to delete said *prpA-D* operon.

3. Claims: 1-11,13,24-33,40-43 (all partially); 16,17,36, 37 (all completely)

Prokaryotic host cells (e.g., of the genus *Escherichia* (e.g., *E. coli*), *Streptomyces*, *Bacillus*, *Pseudomonas* or *Flavobacterium*) which are genetically modified for enhanced synthesis of at least one (complete) polyketide (e.g., 6-dEB), wherein said modification comprises incorporation of an expression system for producing a malonyl-CoA decarboxylase that catalyzes the production of starter and/or extender units.

Said host cells which do not produce polyketide in the absence of genetic modification, and wherein said modification comprises incorporation of an expression system for a polyketide synthase (PKS) and incorporation of an expression system for a phosphopantetheinyl transferase.

A method to produce a polyketide comprising culturing said cells.

A method to assess the results of a procedure effecting modification of PKS genes using said *E. coli* cells.

A method to enhance the production of a polyketide in said microbial host cells which method comprises the provision of said host cell with an expression system for malonyl-CoA decarboxylase, e.g., encoded by the *matA* gene of *R. trifolii*. Recombinant microbial cells (e.g., *Streptomyces* (coelicolor CH999) or *Escherichia* (coli)) that produce a (complete) polyketide (e.g., 6-dEB) which have been modified to contain an expression system for (a) nucleotide sequence(s) encoding said malonyl-CoA decarboxylase, and methods for producing a polyketide using said cells.

A reaction mixture for the production of a polyketide comprising in addition to enzymes catalyzing the production of said polyketide, said malonyl-CoA decarboxylase, e.g., encoded by the *matA* gene from *R. trifolii*, and methods for producing a polyketide using said reaction mixture.

4. Claims: 1-11,13,24-33,40-43 (all partially); 18-23,38, 39 (all completely)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Prokaryotic host cells (e.g., of the genus *Escherichia* (e.g., *E. coli*), *Streptomyces*, *Bacillus*, *Pseudomonas* or *Flavobacterium*) which are genetically modified for enhanced synthesis of at least one (complete) polyketide (e.g., 6-dEB), wherein said modification comprises incorporation of an expression system for producing a malonyl-CoA synthetase that catalyzes the production of starter and/or extender units.

Said host cells which do not produce polyketide in the absence of genetic modification, and wherein said modification comprises incorporation of an expression system for a polyketide synthase (PKS) and incorporation of an expression system for a phosphopantetheinyl transferase. A method to produce a polyketide comprising culturing said cells.

A method to assess the results of a procedure effecting modification of PKS genes using said *E. coli* cells.

A method to enhance the production of a polyketide in said microbial host cells which method comprises the provision of said host cell with an expression system for malonyl-CoA synthetase, e.g., encoded by the *matB* gene of *R. trifolii*, and which possibly further includes providing the substrate (of formula $R_2C(COOH)_2$, with R according to claims 22 and 23) for malonyl-CoA synthetase and an expression system for a second enzyme that effects entry of said substrate into the cell, e.g., the malonate transporter encoded by the *matC* gene of *R. trifolii*.

Recombinant microbial cells (e.g., *Streptomyces* (*coelicolor* CH999) or *Escherichia* (*coli*)) that produce a (complete) polyketide (e.g., 6-dEB) which have been modified to contain an expression system for (a) nucleotide sequence(s) encoding said propionyl-CoA carboxylase, and methods for producing a polyketide using said cells.

A reaction mixture for the production of a polyketide comprising in addition to enzymes catalyzing the production of said polyketide, a first enzyme which catalyzes the conversion of a substrate to an extender or starter unit for said polyketide (malonyl-CoA synthetase), and possibly further a substrate therefor (of formula $R_2C(COOH)_2$, with R according to claim 39), and methods for producing a polyketide using said reaction mixture.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/28573

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9827203 A	25-06-1998	AU 734325 B AU 5701098 A EP 0948613 A JP 2001510993 T US 6258566 B US 6033883 A	07-06-2001 15-07-1998 13-10-1999 07-08-2001 10-07-2001 07-03-2000
WO 9508548 A	30-03-1995	US 5672491 A AU 678058 B AU 7731794 A CA 2171629 A EP 1118665 A EP 0725778 A JP 9505983 T US 6077696 A US 6214573 B US 6215007 B US 5712146 A US 2001024810 A US 5830750 A US 6022731 A US 5843718 A US 5962290 A	30-09-1997 15-05-1997 10-04-1995 30-03-1995 25-07-2001 14-08-1996 17-06-1997 20-06-2000 10-04-2001 10-04-2001 27-01-1998 27-09-2001 03-11-1998 08-02-2000 01-12-1998 05-10-1999
WO 9919518 A	22-04-1999	AU 1094699 A EP 1029076 A	03-05-1999 23-08-2000
WO 9903986 A	28-01-1999	US 6066721 A AU 736972 B AU 8412398 A CN 1267332 T EP 0996714 A HU 0003728 A JP 2001510038 T US 6261816 B US 2001024811 A	23-05-2000 09-08-2001 10-02-1999 20-09-2000 03-05-2000 28-02-2001 31-07-2001 17-07-2001 27-09-2001
WO 0020601 A	13-04-2000	AU 1441500 A EP 1117801 A	26-04-2000 25-07-2001